IMPROVED METHOD FOR PREPARING FLOUR DOUGHS AND PRODUCTS MADE FROM SUCH DOUGHS USING ~~GLYCEROL OXIDASE~~

FIELD OF THE INVENTION

The present invention relates to the field of food manufacturing, in particular to the preparation of improved bakery products and other farinaceous food products. Specifically, the invention concerns the use of glycerol oxidase as a dough strengthening agent and improvement of the quality of baked and dried products made from such improved doughs. There is also provided a method of improving the properties of doughs and baked product by combined use of glycerol oxidase and a lipase.

TECHNICAL BACKGROUND AND PRIOR ART

The "strength" or "weakness" of doughs is an important aspect of making farinaceous finished products from doughs, including baking. The "strength" or "weakness" of a dough is primarily determined by its content of protein and in particular the content and the quality of the gluten protein is an important factor in that respect. Flours with a low protein content is generally characterized as "weak". Thus, the cohesive, extensible, rubbery mass which is formed by mixing water and weak flour will usually be highly extensible when subjected to stress, but it will not return to its original dimensions when the stress is removed.

Flours with a high protein content are generally characterized as "strong" flours and the mass formed by mixing such a flour and water will be less extensible than the mass formed from a weak flour, and stress which is applied during mixing will be restored without breakdown to a greater extent than is the case with a dough mass formed from a weak flour. Strong flour is generally preferred in most baking contexts because of the superior rheological and handling properties

of the dough and the superior form and texture qualities of the finished baked or dried products made from the strong flour dough.

Doughs made from strong flours are generally more stable. Stability of a dough is one of the most important characteristics of flour doughs. Within the bakery and milling industries it is known to use dough "conditioners" to strengthen the dough to increase its stability and strength. Such dough conditioners are normally non-specific oxidizing agents such as e.g. iodates, peroxides, ascorbic acid, K-bromate or azodicarbonamide and they are added to dough with the aims of improving the baking performance of flour to achieve a dough with improved stretchability and thus having a desirable strength and stability. The mechanism behind this effect of oxidizing agents is that the flour proteins, in particular gluten contains thiol groups which, when they become oxidized, form disulphide bonds whereby the protein forms a more stable matrix resulting in a better dough quality and improvements of the volume and crumb structure of the baked products.

However, the use of several of the currently available non-specific oxidizing agents is either objected to by consumers or is not permitted by regulatory bodies. Hence it has been attempted to find alternatives to these conventional flour and dough additives, and the prior art has i.a. suggested the use of glucose oxidase and hexose oxidase for this purpose.

Glycerol oxidase is an oxidoreductase which is capable of oxidizing glycerol. Different types of glycerol oxidase have been described in the literature. Some of these glycerol oxidases need co-factors in order to oxidize glycerol (Shuen-Fu et al., 1996. Enzyme Micro. Technol., **18**:383-387).

However, glycerol oxidase from *Aspergillus japonicus* does not require any co-factors in the oxidation of glycerol to glyceraldehyde (T. Uwajima and O. Terada, 1980. Agri. Biol.

Chem. **44**:2039-2045).

This glycerol oxidase has been characterized by T. Uwajima and O. Terada (Methods in Enzymology, 1982, **89**:243-248) and T. Uwajima et al. (Agric. Biol. Chem., 1979, **43**:2633-2634), and has a pH optimum at 7.0 and K_m and V_{max} are 10.4 mM and 935.6 µmol H_2O_2 min^{-1} respectively using glycerol as substrate. The enzyme is most active on glycerol but also other substrates like dihydroxyacetone, 1,3-propanediol, D-galactose ad D-fructose are oxidized by glycerol oxidase.

Glycerol oxidase not requiring co-factors has also been isolated from *Penicillium* and characterized by Shuen-Fuh Lin et al. (Enzyme Micro. Technol., 1996, **18**:383-387). This enzyme has optimum activity in the pH range from 5.5 to 6.5 at 30°C. The enzyme is stable between 20 and 40°C but loses its activity at temperatures above 50°C.

Other potential sources for glycerol oxidase according to the invention include different fungal species as disclosed in DE-2817087-A, such as *Aspergillus oryzae, Aspergillus parasiticus, Aspergillus flavus, Neurospora crassa, Neurospora sitophila, Neurospora tetrasperma, Penicillium nigricans, Penicillium funiculosum* and *Penicillium janthinellum*.

Glycerol oxidase isolated from the above natural sources has been used for different applications. Thus, glycerol oxidase from *Aspergillus japonicus* has been used for glycoaldehyde production from ethylene glycol (Kimiyasu Isobe and Hiroshi Nishise, 1995, Journal of Molecular Catalysis B: Enzymatic, **1**:37-43). Glycerol oxidase has also been used in the combination with lipoprotein lipase for the determination of contaminated yolk in egg white (Yioshinori Mie, 1996. Food Research International, **29**:81-84). DE-2817087-A and US 4,399,218 disclose the use of glycerol oxidase for the determination of glycerol.

It has now been found that the addition of a glycerol oxidase to a flour dough results in an increased resistance hereof to deformation when the dough is stretched, i.e. this enzyme confers to the dough an increased strength whereby the dough becomes less prone to mechanical deformation. Accordingly, glycerol oxidase is highly useful as a dough conditioning agent in the manufacturing of flour dough based products including not only bread products but also other products made from flour doughs such as noodles and alimentary paste products.

It has also been found that the dough strengthening effect of glycerol oxidase is potentiated significantly when it is combined with a lipase, which in itself does not affect the dough strength. Furthermore, the combined use of glycerol oxidase and lipase results in an improvement of bread quality, in particular in respect of specific volume and crumb homogeneity, which is not a simple additive effect, but reflects a synergistic effect of these two types of enzymes.

SUMMARY OF THE INVENTION

Accordingly, the invention relates in a first aspect to a method of improving the rheological properties of a flour dough and the quality of the finished product made from the dough, comprising adding to the dough 10 to 10,000 units of a glycerol oxidase per kg of flour.

In a further aspect there is provided a method of improving the rheological properties of a flour dough and the quality of the finished product made from the dough, comprising adding to the dough a glycerol oxidase and a lipase.

The invention pertains in a still further aspect to dough improving composition comprising a glycerol oxidase and at least one further dough ingredient or dough additive.

In still further aspects, the invention relates to the use of a glycerol oxidase for improving the rheological properties of a flour dough and the quality of the finished product made from the dough and to the use of a glycerol oxidase and a lipase in combination for improving the rheological properties of a flour dough and the quality of the finished product made from the dough.

DETAILED DISCLOSURE OF THE INVENTION

In one aspect, the present method provides a method of improving the rheological properties of flour doughs.

The expression "rheological properties" as used herein refers particularly to the effects of dough conditioners on dough strength and stability as the most important characteristics of flour doughs. According to American Association of Cereal Chemists (AACC) Method 36-01A the term "stability" can be defined as "the range of dough time over which a positive response is obtained and that property of a rounded dough by which it resists flattening under its own weight over a course of time". According to the same method, the term "response" is defined as "the reaction of dough to a known and specific stimulus, substance or set of conditions, usually determined by baking it in comparison with a control"

As it is mentioned above, it is generally desirable to improve the baking performance of flour to achieve a dough with improved stretchability and thus having a desirable strength and stability by adding oxidizing agents which cause the formation of protein disulphide bonds whereby the protein forms a more stable matrix resulting in a better dough quality and improvements of the volume and crumb structure of baked products.

Thus, the term "rheological properties" relates to the above physical and chemical phenomena which in combination will

determine the performance of flour doughs and thereby also the quality of the resulting products.

The method comprises, as it is mentioned above, the addition of an effective amount of a glycerol oxidase to the dough. It will be understood that the addition can be either to a component of the dough recipe or to the dough resulting from mixing all of the components for the dough. In the present context, "an effective amount" is used to indicate that the amount is sufficient to confer to the dough and/or the finished product improved characteristics as defined herein. Specifically, such an amount is in the range of 10 to 10,000 units of glycerol oxidase per kg flour.

In one useful embodiment of the method according to the invention, the glycerol oxidase can, as it is described in details herein, be isolated from a bacterial species, a fungal species, a yeast species, an animal cell including a human cell or a plant cell. Examples of glycerol oxidase producing fungal species are species belonging to the genera *Aspergillus*, *Neurospora* and *Penicillium*, such as *A. japonicus*, *A. oryzae*, *A. parasiticus*, *A. flavus*, *Neurospora crassa*, *N. sitophila*, *N. tetrasperma*, *Penicillium nigricans*, *P. funiculosum* and *P. janthinellum*.

Glycerol oxidase can be derived as a native enzyme from natural sources such as the above.

It is one objective of the invention to provide improved bakery products. In accordance with the invention, a bakery product dough including a bread dough is prepared by mixing flour with water, a leavening agent such as yeast or a conventional chemical leavening agent, and an effective amount of glycerol oxidase under dough forming conditions. It is, however, within the scope of the invention that further components can be added to the dough mixture.

Typically, such further dough components include conventio-

nally used dough components such as salt, sweetening agents such as sugars, syrups or artificial sweetening agents, lipid substances including shortening, margarine, butter or an animal or vegetable oil, glycerol and one or more dough additives such as emulsifying agents, starch degrading enzymes, cellulose or hemicellulose degrading enzymes, proteases, lipases, non-specific oxidizing agents such as those mentioned above, flavouring agents, lactic acid bacterial cultures, vitamins, minerals, hydrocolloids such as alginates, carrageenans, pectins, vegetable gums including e.g. guar gum and locust bean gum, and dietary fiber substances.

Conventional emulsifying agents used in making flour dough products include as examples monoglycerides, diacetyl tartaric acid esters of mono- and diglycerides of fatty acids, and lecithins e.g. obtained from soya. Among starch degrading enzymes, amylases are particularly useful as dough improving additives. Other useful starch degrading enzymes which may be added to a dough composition include glucoamylases and pullulanases. In the present context, further interesting enzymes are xylanases and oxidoreductases such as glucose oxidase, pyranose oxidase, hexose oxidase, sulfhydryl oxidase, and lipases.

A preferred flour is wheat flour, but doughs comprising flour derived from other cereal species such as from rice, maize, barley, rye and durra are also contemplated.

In accordance with the invention, the dough is prepared by admixing flour, water, the glycerol oxidase and optionally other ingredients and additives. The glycerol oxidase can be added together with any dough ingredient including the water or dough ingredient mixture or with any additive or additive mixture. The dough can be prepared by any conventional dough preparation method common in the baking industry or in any other industry making flour dough based products.

The glycerol oxidase can be added as a liquid preparation or in the form of a dry powder composition either comprising the enzyme as the sole active component or in admixture with one or more other dough ingredients or additive.

The amount of the glycerol oxidase added is an amount which results in the presence in the dough of 10 to 5,000 units (as defined in the following) such as 10 to 2,500 units per kg of flour. In useful embodiments, the amount is in the range of 20 to 1,500 units per kg of flour.

The effect of the glycerol oxidase on the rheological properties of the dough can be measured by standard methods according to the International Association of Cereal Chemistry (ICC) and the American Association of Cereal Chemistry (AACC) including the amylograph method (ICC 126), the farinograph method (AACC 54-21) and the extensigraph method (AACC 54-10). The AACC method 54-10 defines the extensigraph in the following manner: "the extensigraph records a load-extension curve for a test piece of dough until it breaks. Characteristics of load-extension curves or extensigrams are used to assess general quality of flour and its responses to improving agents". In effect, the extensigraph method measures the relative strength of a dough. A strong dough exhibits a higher and, in some cases, a longer extensigraph curve than does a weak dough.

In a preferred embodiment of the method according to the invention, the resistance to extension of the dough in terms of the ratio between the resistance to extension (height of curve, B) and the extensibility (length of curve, C), i.e. the B/C ratio as measured by the AACC method 54-10 is increased by at least 10% relative to that of an otherwise similar dough not containing glycerol oxidase. In more preferred embodiments, the resistance to extension is increased by at least 20%, such as at least 50% and in particular by at least 100%.

It has been found that the addition of glycerol oxidase to bakery product doughs results in bakery products such as yeast leavened and chemically leavened products in which the specific volume is increased relative to an otherwise similar bakery product, prepared from a dough not containing glycerol oxidase. In this context, the expression "specific volume" is used to indicate the ratio between volume and weight of the product. It has been found that, in accordance with the above method, the specific volume can be increased significantly such as by at least 10%, preferably by at least 20%, including by at least 30%, preferably by at least 40% and more preferably by at least 50%.

The method according to the invention is highly suitable for improving the rheological properties and quality of the finished products of conventional types of yeast leavened bread products based on wheat flour, such as loaves and rolls. The method is also suitable for improving the rheological properties of doughs containing chemical leavening agents (baking powder) and the quality of products made from such doughs. Such product include as examples sponge cakes and muffins.

In one interesting aspect, the invention is used to improve the rheological properties of doughs intended for noodle products including "white noodles" and "chinese noodles" and to improve the textural qualities of the finished noodle products. A typical basic recipe for the manufacturing of noodles comprises the following ingredients: wheat flour 100 parts, salt 0.5 parts and water 33 parts. Furthermore, glycerol is often added to the noodle dough. The noodles are typically prepared by mixing the ingredients in an appropriate mixing apparatus followed by rolling out the noodle dough using an appropriate noodle machine to form the noodle strings which are subsequently air dried.

The quality of the finished noodles is assessed i.a. by their

colour, cooking quality and texture. The noodles should cook as quickly as possible, remain firm after cooking and should preferably not loose any solids to the cooking water. On serving the noodles should preferably have a smooth and firm surface not showing stickiness and provide a firm "bite" and a good mouthfeel. Furthermore, it is important that the white noodles have a light colour.

Since the appropriateness of wheat flour for providing noodles having the desired textural and eating qualities may vary according to the year and the growth area, it is usual to add noodle improvers to the dough in order to compensate for sub-optimal quality of the flour. Typically, such improvers will comprise dietary fiber substances, vegetable proteins, emulsifiers and hydrocolloids such as e.g. alginates, carrageenans, pectins, vegetable gums including guar gum and locust bean gum, and amylases, and as mentioned above, glycerol.

It is therefore an important aspect of the invention that the glycerol oxidase according to the invention is useful as a noodle improving agent optionally in combination with glycerol and other components currently used to improve the quality of noodles. Thus, it is contemplated that noodles prepared in accordance with the above method will have improved properties with respect to colour, cooking and eating qualities including a firm, elastic and non-sticky texture and consistency.

In a further useful embodiment, the dough which is prepared by the method according to the invention is a dough for preparing an alimentary paste product. Such products which include as examples spaghetti and maccaroni are typically prepared from a dough comprising main ingredients such as flour, eggs or egg powder and/or water. After mixing of the ingredient, the dough is formed to the desired type of paste product and air dried. It is contemplated that the addition of glycerol oxidase to a paste dough, optionally in combina-

tion with glycerol, will have a significant improving effect on the extensibility and stability hereof resulting in finished paste product having improved textural and eating qualities.

In a useful embodiment, there is provided a dough improving method wherein at least one further enzyme is added to the dough ingredient, dough additive or the dough. In the present context, suitable enzymes include cellulases, hemicellulases, xylanases, starch degrading enzymes, oxidoreductases and proteases.

In a further aspect, the invention relates to a method of improving the rheological properties of a flour dough and the quality of the finished products made from the dough which comprises that both a glycerol oxidase and a lipase is added to the dough.

It was surprisingly found that the two types of enzymes were capable of interacting with each other under the dough conditions to an extent where the effect on improvement of the dough strength and bread quality by the enzymes was not only additive, but the effect was synergistic.

Thus, with respect to improvement of dough strength it was found that with glycerol oxidase alone, the B/C ratio as measured after 45 minutes of resting was increased by 34%, with lipase alone no effect was observed. However, when combining the two enzymes, the B/C ratio was increased by 54%, i.e. combining the glycerol oxidase with the lipase enhanced the dough strengthening effect of glycerol oxidase by more than 50%. Thus, one objective of combining glycerol oxidase and a lipase is to provide an enhancement of the dough strengthening effect of glycerol oxidase by at least 25% such as at least 50% including at least 75%, determined as described herein.

In relation to improvement of finished product, it was found

that the combined addition of glycerol oxidase and a lipase resulted in a substantial synergistic effect in respect to crumb homogeneity as defined herein. Also, with respect to the specific volume of baked product a synergistic effect was found. Thus, for a bread product, the addition of lipase alone typically results in a negligible increase of the specific volume, addition of glycerol oxidase alone in an increase of about 25%, whereas a combined addition of the two enzymes results in an increase of more than 30%.

Further in relation to improvement of the finished product, it was found that the addition of lipase resulted in modification of the glycolipids, monogalactosyl diglyceride and digalactosyl diglyceride present in dough. These components were converted to the more polar components monogalactosyl monoglyceride and digalactosyl monoglyceride. As galactosyl monoglycerides are more surface active components than galactosyl diglycerides it is assumed that galactosyl monoglycerides contributed to the observed improved crumb cell structure and homogeneity. Thus, one objective of using lipase is to hydolyse at least 10% of the galactosyl diglycerides normally present in a flour dough to the corresponding galactosyl monoglycerides, such as at least 50% including at least 100%.

The details of such a method using combined addition of glycerol oxidase and lipase are, apart from the use of a lipase in combination with glycerol oxidase, substantially similar to those described above for a method according to the invention which does not require the addition of a lipase.

When using, in accordance with the invention, a lipase in combination with a glycerol oxidase, the amount of lipase is typically in the range of 10 to 100,000 lipase units (LUS) (as defined in the following) per kg flour including the range of 10 to 20,000 LUS, e.g. 100 to 15,000 LUS such as 500 to 10,000 LUS.

Lipases that are useful in the present invention can be derived from a bacterial species, a fungal species, a yeast species, an animal cell and a plant cell. Whereas the enzyme may be provided by cultivating cultures of such source organisms naturally producing lipase, it may be more convenient and cost-effective to produce it by means of genetically modified cells such as it is described in details in the following examples. In the latter case, the term "derived" may imply that a gene coding for the lipase is isolated from a source organism and inserted into a host cell capable of expressing the gene.

Thus, the enzyme may in a useful embodiment be derived from an *Aspergillus* species including as examples *A. tubigensis*, *A. oryzae* and *A. niger*.

Presently preferred lipases include the lipase designated Lipase 3, the production and characteristics of which is described in details in the following examples, or a mutant of this enzyme. In the present context, the term "mutant" refers to a lipase having, relative to the wild-type enzyme, an altered amino acid sequence. A further preferred lipase is the lipase found in the commercial product, GRINDAMYL™ EXEL 16.

In a further aspect of the invention there is provided a dough improving composition comprising a glycerol oxidase and at least one further dough ingredient or dough additive.

The further ingredient or additive can be any of the ingredients or additives which are described above. The composition may conveniently be a liquid preparation comprising the glycerol oxidase. However, the composition is conveniently in the form of a dry composition.

The amount of the glycerol oxidase in the composition is in the range of 10 to 10,000 units per kg flour. It will be

appreciated that this indication of the amount of enzyme implies that a recommended appropriate amount of the composition will result in the above stated amount in the dough to which it is added. In specific embodiments, the amount of glycerol oxidase is in the range of 10 to 5,000 units such as 10 to 2,500 units per kg of flour. In other useful embodiments, the amount is in the range of 20 to 1,500 units per kg of flour.

In another embodiment, the dough improving composition may further comprises a lipase as defined above and in the amounts as also described above in relation to the method according to the invention.

Optionally, the composition is in the form of a complete dough additive mixture or pre-mixture for making a particular finished product and containing all of the dry ingredients and additives for such a dough. In specific embodiments, the composition is one particularly useful for preparing a baking product or in the making of a noodle product or an alimentary paste product.

In one advantageous embodiment of the above method at least one further enzyme is added to the dough. Suitable examples hereof include a cellulase, a hemicellulase, a xylanase, a starch degrading enzyme, hexose oxidase and a protease.

In a preferred advantageous embodiment, the further added enzyme is a lipase. It has been found that in accordance with the above method, the crumb homogeneity and specific volume of the bakery product can be increased significantly as compared to that of an otherwise similar bakery product prepared from a dough not containing glycerol oxidase, and from a similar bakery product prepared from a dough containing glycerol oxidase.

In a still further aspect, the present invention pertains to the use of a glycerol oxidase and a lipase in combination for

improving the rheological properties of a flour dough and the quality of the finished product made from the dough.

In this connection, specific embodiments include use wherein the improvement of the rheological properties of the dough include that the resistance to extension of the dough in terms of the ratio between resistance to extension (height of curve, B) and the extensibility (length of curve, C), i.e. the B/C ratio, as measured by the AACC method 54-10 is increased by at least 10% relative to that of an otherwise similar dough that does not contain glycerol oxidase and use wherein the improvement of the quality of the finished product made from the dough is that the average pore diameter of the crumb of the bread made from the dough is reduced by at least 10%, relative to a bread which is made from a bread dough without addition of the lipase.

In a further embodiment, the use according to the invention, implies that the improvement of the quality of the finished product made from the dough consists in that the pore homogeneity of the crumb of the bread made from the dough is increased by at least 5%, relative to a bread which is made from a bread dough without addition of the lipase. The pore homogeneity of bread is conveniently measured by means of an image analyzer composed of a standard CCD-video camera, a video digitiser and a personal computer with WinGrain software. Using such an analyzer, the results of pore diameter in mm and pore homogeneity can be calculated as an average of measurements from 10 slices of bread. The pore homogeneity is expressed in % of pores that are larger than 0.5 times the average of pore diameter and smaller than 2 times the average diameter.

In a further embodiment, the use relates to improvement of the rheological characteristics of the dough including that the gluten index (as defined hereinbelow) in the dough is increased by at least 5%, relative to a dough without addition of a lipase, the gluten index is determined by means

of a Glutomatic 2200 apparatus.

BRIEF DESCRIPTION OF THE FIGURES

The present invention is further illustrated by reference to the accompanying figures in which

Fig. 1 shows the restriction map of the genomic clone of the *lip*A gene,

Fig. 2 shows the structure of the *lip*A gene encoding lipase 3,

Fig. 3 shows a chromatogram of HIC fractionated culture supernatant of an *Aspergillus tubigensis* transformant with 62-fold increase of lipase 3, and

Fig. 4 shows a chromatogram of HIC fractionated culture supernatant of the untransformed *Aspergillus tubigensis* strain.

The invention will now be described by way of illustration in the following non-limiting examples.

A. PRODUCTION AND PURIFICATION OF GLYCEROL OXIDASE (GLOX)

EXAMPLE 1

Production, extraction and purification of glycerol oxidase using different strains and cultivation conditions

1. Production, extraction and purification of glycerol oxidase using *Aspergillus japonicus* ATCC 1042 cultivated in a production medium containing 3% glycerol

The following assay for determination of glycerol oxidase activity was used:

The assay is based on the method described by Sullivan and Ikawa (Biochimica and Biophysica Acta, 1973, **309**:11-22), but modified as described in the following. An assay mixture containing 150 µl 2% glycerol (in 100 mM phosphate buffer, pH 7.0), 120 µl 100 mM phosphate buffer, pH 7.0, 10 µl o-dianisidin dihydrochloride (Sigma D 3252, 3 mg/ml in H_2O), 10 µl peroxidase (POD) (Sigma P8125, 0.1 mg/ml in 100 mM phosphate buffer, pH 7.0) and 10 µl glycerol oxidase (GLOX) solution. The controls are made by adding buffer in place of GLOX solution. The incubation is started by the addition of glycerol. After 15 minutes of incubation at 25°C in micro-titer plates, the absorbance at 402 nm is read in a Elisa reader. A standard curve is constructed using varying concentrations of H_2O_2 in place of the enzyme solution. The reaction can be described in the following manner:

$$\text{Glycerol} + O_2 \xrightarrow{\text{GLOX}} \text{glyceraldehyde} + H_2O_2$$

$$H_2O_2 + \text{o-dianisidine}_{red} \xrightarrow{\text{POD}} 2\ H_2O + \text{o-dianisidine}_{ox}$$

Oxidised o-dianisidine has a yellow colour absorbing at 402 nm.

One glycerol oxidase unit (U) is the amount of enzyme which catalyses the production of 1 µmole H_2O_2 per minute at 25°C, pH 7.0 at a substrate concentration of 0.2 M glycerol.

A spore suspension of *Aspergillus japonicus* ATCC 1042 was prepared by incubating *A.japonicus* on PDA medium (30°C, 7 days) and washing with 10 ml of 0.2% Tween 80. A preculture was prepared by inoculating 1 ml of the resulting spore suspension in 300 ml production medium containing 3.0 % of glycerol (87%, Merck), 0.3% of yeast extract (Difco), 0.1% of meat extract (Difco), 0.1 % KH_2PO_4 (Merck), 0.1% of $MGSO_4$ *

$7H_2O$ (Merck), 0.1% antifoam (Contra spum) and 70 mg/l of chloramphenicolum (Mecobenzon) (pH adjusted to 7.2 with NaOH) in a 500 ml flask. The preculture was incubated overnight at 30°C with shaking (200 rpm).

A 30 litre fermenter with 15 litre production medium was inoculated with 900 ml (corresponding to 3 flasks) of the resulting overnight preculture, and cultured at 30°C for 25 hours under continuous stirring (350 rpm) and aeration (15 l/min). After culturing, the mycelia was harvested from the resulting culture broth by filtration on a Whatman GF/B filter by suction, and washed with 3 litres of deionized water. The mycelium yield was 186 g (wet weight).

A part (50 g) of the resulting mycelial mat was suspended in 700 ml of 50 mM borate buffer (pH 10.0), and disrupted by ultrasonication (Branson, Sonifer 250) at 5°C (3 x 5 minutes). After disruption, the mycelia was removed by centrifugation (29,000 g for 15 minutes), the cell-free extract (700 ml) was brought to 40% saturation with ammonium sulfate and the resulting precipitate was removed by centrifugation (29,000 g for 20 minutes). The ammonium sulfate concentration was then increased to 70% saturation to precipitate the enzyme. The resulting precipitate was collected and solubilized in 100 ml of 50 mM borate buffer (pH 10.0). The crude extract was then dialysed for 24 hours against 5 l of 50 mM borate buffer (pH 10.0). After dialysis the insoluble matters in the crude extract were removed by centrifugation (18,000 x g for 10 minutes). The resulting supernatant contained 8.7 units of glycerol oxidase activity per ml.

2. Production, extraction and purification of glycerol oxidase using *Aspergillus japonicus* ATCC 1042 cultivated in a production medium containing 5% glycerol

A spore suspension of *Aspergillus japonicus* ATCC 1042 was

prepared as described above. A preculture was prepared by inoculating 1 ml of the resulting spore suspension into a flask (500 ml) containing 200 ml production medium (5.0 % glycerol, 0.25% yeast extract, 0.1% Malt extract, 0.7% anti-foam (Contra spum), pH adjusted to 6.2 with HCl, sterilization at 121°C for 90 minutes). The preculture was incubated 3 days at 30°C with continuous shaking (200 rpm).

A 6 litre fermenter with 5 litre production medium as described above was inoculated with 50 ml of the resulting preculture and cultured at 30°C for 3 days under continuous stirring (250 rpm) and aeration (5 l/min). After culturing the mycelia was harvested from the resulting culture broth by filtration on a Whatman GF/B filter by suction, and washed with 3 litre ionized water containing 0.9% NaCl.

The resulting mycelia mat was frozen in liquid nitrogen, suspended in 200 ml of 50 mM phosphate buffer (pH 7.0) and disrupted by ultrasonication (Branson, Sonifer 250) at 5°C (4 minutes). After disruption, the mycelia was removed by filtration on a Whatman GF/A filter by suction. The enzyme in the resulting filtrate was concentrated on a AMICON® 8400 ultrafiltration unit and contained 87 units of glycerol oxidase per ml after ultrafiltration.

3. Production, extraction and purification of glycerol oxidase using *Aspergillus japonicus* ATCC 1042 cultivated in a production medium containing 10% glycerol

A spore suspension of *Aspergillus japonicus* ATCC 1042 was prepared as described above. A 1 ml sample of the resulting spore suspension was inoculated into each of 5 flasks (500 ml) with 200 ml production medium containing 10.0 % of glycerol, 0.1% of yeast extract and 0.1% of malt extract (pH adjusted to 6.2 with HCl, sterilization at 121°C for 15 minutes). The cultures were incubated for 5 days at 30°C with shaking (140 rpm).

The extraction and concentration of the enzyme was carried

out as described above. The resulting filtrate contained 66 units of glycerol oxidase per ml after ultrafiltration.

4. Production of glycerol oxidase from *Penicillium funiculosum* and *Penicillium janthinellum*

Spore suspensions of *Penicillium funiculosum* NRRL 1132 and *Penicillium janthinellum* NRRL 2016 were prepared as described above. A 1 ml sample of each of the resulting spore suspensions was inoculated into separate flasks (1000 ml) containing 100 g wheat bran and 100 ml water (two flasks for each culture)

Glycerol oxidase was extracted by suspending the wheat bran cultures in 900 ml of 30 mM phosphate buffer (pH 6.5) containing 0.1% Triton X100 (Merck). The mycelial mat was removed from the cultivation media by filtration using a Whatman GF/B filter. The resulting mycelia mat was frozen in liquid nitrogen, suspended in 200 ml of 50 mM phosphate buffer (pH 7.0) and disrupted by ultrasonication (Branson, Sonifer 250) at 5°C (4 minutes). After disruption, the mycelia was removed by filtration on a Whatman GF/A filter by suction. The resulting filtrate from the *Penicillium funiculosum* culture contained 7.4 units of glycerol oxidase per ml, and the resulting filtrate from the *Penicillium janthinellum* culture contained 11.3 units of glycerol oxidase per ml.

B. PRODUCTION, PURIFICATION AND CHARACTERIZATION OF *ASPERGILLUS TUBIGENSIS* LIPASE 3

Materials and Methods

(i) Determination of lipase activity and protein

1. Plate assay on tributyrin-containing medium

The assay is modified from Kouker and Jaeger (Appl. Environ. Microbiol., 1987, **53**:211-213).

A typical protocol for this assay is as follows: 100 ml 2% agar in 50 mM sodium phosphate buffer (pH 6.3) is heated to boiling, and after cooling to about 70°C under stirring, 5 ml 0.2% Rhodamine B is added under stirring plus 40 ml of tributyrin. The stirring is continued for 2 minutes. The mixture is then sonicated for 1 minute. After an additional 2 minutes of stirring, 20 ml of the agar mixture is poured into individual petri dishes. In the absence of lipase activity, the agar plates containing tributyrin and Rhodamine B will appear opaque and are pink coloured.

To quantify lipase activity, holes having a diameter of 3 mm are punched in the above agar and filled with 10 µl of lipase preparation. The plates are incubated for varying times at 37°C. When lipase activity is present in the applied preparation to be tested, a sharp pink/reddish zone is formed around the holes. When the plates are irradiated with UV light at 350 nm, the lipase activity is observed as halos of orange coloured fluorescence.

2. Modified Food Chemical Codex assay for lipase activity

Lipase activity based on hydrolysis of tributyrin is measured according to Food Chemical Codex, Forth Edition, National Academy Press, 1996, p. 803. With the modification that the pH is 5.5 instead of 7. One LUT (lipase unit tributyrin) is defined as the amount of enzyme which can release 2 µmol butyric acid per min. under the above assay conditions.

3. *p*-nitrophenyl acetate assay

Lipase activity can also be determined colorimetrically using *p*-nitrophenyl acetate as a substrate e.g. using the following protocol: In a microtiter plate 10 µl of sample or blank is added followed by the addition of 250 µl substrate (0.5 mg *p*-nitrophenyl acetate per ml 50 mM phosphate buffer, pH 6.0).

The microtiter plate is incubated for 5 minutes at 30°C and the absorbance at 405 nm is read using a microplate reader. 1 unit is defined as 1 µmol *p*-nitrophenol released per 5 minutes.

4. *p*-nitrophenyl hexanoate assay

Lipase activity can be determined by using *p*-nitrophenyl hexanoate as a substrate. This assay is carried out by adding 10 µl of sample preparation or blank to a microtiter plate followed by the addition of 250 µl substrate (0.5 mg *p*-nitrophenyl hexanoate per ml of 20 mM phosphate buffer, pH 6.). At this concentration of substrate the reaction mixture appears as a milky solution. The microtiter plate is incubated for 5 minutes at 30°C and the absorbance at 405 nm is read in a microplate reader.

5. Titrimetric assay of lipase activity

Alternatively, lipase activity is determined according to Food Chemical Codex (3rd Ed., 1981, pp 492-493) modified to sunflower oil and pH 5.5 instead of olive oil and pH 6.5. The lipase activity is measured as LUS (lipase units sunflower) where 1 LUS is defined as the quantity of enzyme which can release 1 µmol of fatty acids per minute from sunflower oil under the above assay conditions.

6. Protein measurement

During the course of purification of lipase as described in the following, the protein eluted from the columns was measured by determining absorbance at 280 nm. The protein in the pooled samples was determined in microtiter plates by a sensitive Bradford method according to Bio-Rad (Bio-Rad Bulletin 1177 EG, 1984). Bovine serum albumin was used as a standard.

EXAMPLE 2

Production, purification and characterization of lipase 3

2.1. Production

A mutant strain of *Aspergillus tubigensis* was selected and used for the production of wild type lipase. This lipase is referred to herein as lipase 3. The strain was subjected to a fermentation in a 750 l fermenter containing 410.0 kg of tap water, 10.8 kg soy flour, 11.1 kg ammonium monohydrogenphosphate, 4.0 kg phosphoric acid (75%), 2.7 kg magnesium sulfate, 10.8 kg sunflower oil and 1.7 kg antifoam 1510. The substrate was heat treated at 121°C for 45 minutes. The culture media was inoculated directly with 7.5×10^9 spores of the mutant strain. The strain was cultivated for three days at 38°C, pH controlled at 6.5, aeration at 290 l/min and stirring at 180 rpm the first two days and at 360 rpm the last day. The fermentate was separated using a drum filter and the culture filtrate was concentrated 3.8 times by ultrafiltration. The concentrated filtrate was preserved with potassium sorbate (0.1%) and sodium benzoate (0.2%) and used as a starting material for purification of lipase.

2.2. Purification of lipase

A 60 ml sample of ferment (cf. 2.1) containing 557 LUS/ml, pH 5.5 was first filtered through a GF/B filter and subsequently through a 0.45 µm filter. The filtered sample was desalted using a Superdex G25 SP column (430 ml, 22 x 5 cm) equilibrated in 20 mM triethanolamine, pH 7.3. The flow rate was 5 ml/min. The total volume after desalting was 150 ml.

The desalted sample was applied to a Source Q30 anion exchanger column (100 ml, 5x5 cm) equilibrated in 20 mM triethanolamine, pH 7.3. The column was washed with equilibration buffer until a stable baseline was obtained.

Lipase activity was eluted with a 420 ml linear gradient from 0 to 0.35 M sodium chloride in equilibration buffer, flow rate 5 ml/min. Fractions of 10 ml were collected. Sodium acetate (100 µl of a 2M solution) was added to each fraction to adjust pH to 5.5. Fractions 26-32 (70 ml) were pooled.

To the pool from the anion exchange step was added ammonium sulfate to 1 M and the sample was applied to a Source Phenyl HIC column (20 ml, 10x2 cm) equilibrated in 20 mM sodium acetate (pH 5.5), 1 M ammonium sulfate. The column was washed with the equilibration buffer. Lipase was eluted with a 320 ml linear gradient from 1 M to 0 M ammonium sulfate in 20 mM sodium acetate (pH 5.5), flow 1.5 ml/min. Fractions of 7.5 ml were collected.

Fractions 33-41 were analyzed by SDS-PAGE using a NOVEX system with precast gels. Both electrophoresis and silver staining of the gels were done according to the manufacturer (Novex, San Diego, USA). (The same system was used for native electrophoresis and isoelectric focusing). It was found that fraction 40 and 41 contained lipase as the only protein.

2.3. Characterization of the purified lipase

(i) Determination of molecular weight

The apparent molecular weight of the native lipase was 37.7 kDa as measured by the above SDS-PAGE procedure. The purified lipase eluted at a molecular weight of 32.2 kDa from a Superose 12 gel filtration column (50 mM sodium phosphate, 0.2 M sodium chloride, pH 6.85, flow 0.65 ml/min) and is therefore a monomer.

The molecular weight of the lipase was also determined by matrix-assisted laser desorption ionisation (MALDI) by means of a time-of-flight (TOF) mass spectrometer (Voyager Bio-Spectrometry Workstation, Perspective Biosystems). Samples were prepared by mixing 0.7 µl of desalted lipase solution

and 0.7 µl of a matrix solution containing sinapic acid (3.5-dimethoxy-4-hydroxy cinnamic acid) in 70% acetonitrile (0.1% TFA, 10 mg/ml). 0.7 µl of the sample mixture was placed on top of a stainless steel probe tip and allowed to air-dry prior to introduction into the mass spectrometer. Spectra were obtained from at least 100 laser shots and averaged to obtain a good signal to noise ratio. The molecular mass for the lipase was found to be 30,384 Da and 30,310 Da by two independent analyses.

Digestion of the lipase with endo-β-N-acetyl-glucosamidase H (10 µl) from *Streptomyces* (Sigma) was carried out by adding 200 µl lipase and incubating at 37°C for 2 hours. The digestion mixture was desalted using a VSWP filter and analyzed directly by MALDI mass spectrometry. A major component of deglycosylated lipase gave a mass of 29,339 Da and 29,333 Da by two independent analyses. A minor component with a mass of 29,508 Da was also observed. These values corresponds well to the later calculated theoretical value of 28,939 Da based on the complete amino acid sequence of the mature lipase.

(ii) Determination of the isoelectric point

The isoelectric point (pI) for the lipase was determined by isoelectric focusing and was found to be 4.1.

A calculation of the pI based on the amino acid sequence as determined in the following and shown as SEQ ID NO: 9 gave an estimated pI of 4.07.

(iii) Determination of temperature stability

Eppendorf tubes with 25 µl of purified lipase 3 plus 50 µl 100 mM sodium acetate buffer (pH 5.0) were incubated for 1 hour in a water bath at respectively 30, 40, 50, and 60°C. A control was treated in the same way, but left at room temperature. After 1 hour the lipase 3 activity was determined by the p-nitrophenyl acetate assay as described above.

The purified lipase had a good thermostability. It was found that the lipase maintained 60% of its activity after 1 hour at 60°C. 80% and 85% activity was maintained after 1 hour at 50°C and 40°C respectively.

(iv) Determination of pH stability

Purified lipase 3 (200 µl) was added to 5 ml of 50 mM buffer solutions: (sodium phosphate, pH 8.0, 7.0 and 6.0 and sodium acetate pH 5.0, 4.0 and 3.5). The control was diluted in 5 ml of 4 mM sodium acetate pH 5.5. After four days at room temperature the residual activity was measured by the Modified Food Chemical Codex assay for lipase activity as described above. The lipase was very stable in the pH range from 4.0 to 7.0 where it maintained about 100% activity relative to the control (Table 2.1). At pH 3.5 the lipase maintained 92% activity, and at pH 8.0 95% residual activity was maintained as compared to the control.

Table 2.1. pH stability of lipase 3

pH	Activity (LUT/ml)	Activity (%)
Control (pH 5.5)	89.2	100
3.5	82.5	92
4.0	91.7	103
5.0	86.5	97
6.0	92.4	104
7.0	90.6	102
8.0	84.4	95

EXAMPLE 3

Amino acid sequencing of lipase 3

Purified lipase enzyme was freeze-dried and 100 µg of the freeze-dried material was dissolved in 50 µl of a mixture of 8 M urea and 0.4 M ammonium hydrogencarbonate, pH 8.4. The dissolved protein was denatured and reduced for 15 minutes at 50°C following overlay with nitrogen and addition of 5 µl 45 mM dithiothreitol. After cooling to room temperature, 5 µl of 100 mM iodoacetamide was added for the cysteine residues to be derivatized for 15 minutes at room temperature in the dark under nitrogen.

135 µl of water and 5 µg of endoproteinase Lys-C in 5 µl of water was added to the above reaction mixture and the digestion was carried out at 37°C under nitrogen for 24 hours. The resulting peptides were separated by reverse phase HPLC on a VYDAC C18 column (0.46 x 15 cm; 10 µm; The Separation Group, California, USA) using solvent A: 0.1% TFA in water and solvent B: 0.1% TFA in acetonitrile. Selected peptides were rechromatographed on a Develosil C18 column (0.46 x 10 cm, Novo Nordisk, Bagsværd, Denmark) using the same solvent system, prior to N-terminal sequencing. Sequencing was done using an Applied Biosystems 476A sequencer using pulsed-liquid fast cycles according to the manufacturer's instructions (Applied Biosystems, California, USA).

For direct N-terminal sequencing, the purified protein was passed through a Brownlee C2 Aquapore column (0.46 x 3 cm, 7 µm, Applied Biosystems, California, USA) using the same solvent system as above. N-terminal sequencing was then performed as described above. As the protein was not derivatized prior to sequencing, cysteine residues could not be determined.

The following peptide sequences were found:

N-terminal: Ser-Val-Ser-Thr-Ser-Thr-Leu-Asp-Glu-Leu-Gln-Leu-Phe-Ala-Gln-Trp-Ser-Ala-Ala-Ala-Tyr-X-Ser-Asn-Asn
(SEQ ID NO:1)

Internal peptide 1: Val-His-Thr-Gly-Phe-Trp-Lys
(SEQ ID NO:2)

Internal peptide 2: Ala-Trp-Glu-Ser-Ala-Ala-Asp-Glu-Leu-Thr-Ser-Lys-Ile-Lys
(SEQ ID NO:3)

No further peptides could be purified from the HPLC fractionation presumably because they were very hydrophobic and therefore tightly bound to the reverse phase column.

A search in SWISS-PROT database release 31 for amino acid sequences with homology to the above peptides was performed and only three sequences were found.

All of the above peptides showed a low homology to the above known sequences. Especially internal peptide 2 has very low homology to the three lipases, LIP-RHIDL, LIP-RHIMI and MDLA-PENCA from *Rhizopus delamar* (Haas and Berka, Gene, 1991, **109**:107-113), *Rhizomucor miehei* (Boel et al., Lipids, 1988, **23**:701-706) and *Penicillium camenbertii* (Yamaguchi et al., Gene, 1991, **103**:61-67; Isobe and Nokihara, Febs. Lett., 1993, **320**:101-106) respectively. Although the homology was not very high it was possible to position the lipase 3 peptides on these sequences as it is shown in the below Table 3.1.

Table 3.1. Alignment of lipase 3 peptides with known lipase sequences

```
LIP_RHIDL    MVSFISISQGVSLCLLVSSMMLGSSAVPVSGKSGSSNTAVSASDNAALPP    50
LIP_RHIMI    MVLKQRANYLGFLIVFFTAFLV--EAVPIKRQSNSTVDS--------LPP    40
MDLA_PENCA   MRLS-----------FFTAL-----------------SAVASLGYALPG    21
             *                .  ...                .        **
```

```
     N-Terminal                SVSTSTLDELQLFAQWSAAAYXSNN

     LIP_RHIDL   LISSRCAPPSNKGSKSDLQAEPYNMQKNTEWYESHGGNLTSIGKRDDNLV   100
     LIP_RHIMI   LIPSRTSAPSSSPSTTDPEAPAM----------SRNGPLPS----DVETK    76
  5  MDLA_PENCA  KLQSR------DVSTSELDQFEFWVQYAAASY------------------    47
                  . **       . *... ..

     LIP_RHIDL   GGMTLDLPSDAPPISLSSSTNSASDGGKVVAATTAQIQEFTKYAGIAATA   150
     LIP_RHIMI   YGMALNATSYPDSV-----VQAMSIDGGIRAATSQEINELTYYTTLSANS   121
 10  MDLA_PENCA  ----------------------------------YEADYTAQVGDKL    60
                                                    * .  . ....

     LIP_RHIDL   YCRSVVPGNKWDCVQCQKWVPDGKIITTFT-SLLSDTNGYVLRSDKQKTI   199
     LIP_RHIMI   YCRTVIPGATWDCIHCDA-TEDLKIIKTWS-TLIYDTNAMVARGDSEKTI   169
 15  MDLA_PENCA  SCSKG------NCPEVEA--TGATVSYDFSDSTITDTAGYIAVDHTNSAV   102
                  *..       .* . .    . ..  ... . . **.. .  .......

     Peptide 1                                 VHTGFWK
     Peptide 2                                          AWESAADELTSK
 20
     LIP_RHIDL   YLVFRGTNSFRSAITDIVFNFSDYKPVKGAKVHAGFLSSYEQVVNDYFPV   249
     LIP_RHIMI   YIVFRGSSSIRNWIADLTFVPVSYPPVSGTKVHKGFLDSYGEVQNELVAT   219
     MDLA_PENCA  VLAFRGSYSVRNWVADATFVHTNPGLCDGCLAELGFWSSWKLVRDDIIKE   152
                  ..***. * *. ..* .*   .    .*  .. ** ...  . ..
 25
     Peptide 2   IK

     LIP_RHIDL   VQEQLTAHPTYKVIVTGHSLGGAQALLAGMDLYQREPRLSPKNLSIFTVG   299
     LIP_RHIMI   VLDQFKQYPSYKVAVTGHSLGGATALLCALDLYQREEGLSSSNLFLYTQG   269
 30  MDLA_PENCA  LKEVVAQNPNYELVVVGHSLGAAVATLAATDL--RGKGYPSAKLYAYA--   198
                  . .   . *.*.. *.*****.* * * . **  *.   .. .*  ..

     LIP_RHIDL   GPRVGNPTFAYYVESTGIPFQRTVHKRDIVPHVPPQSFGFLHPGVESWIK   349
     LIP_RHIMI   QPRVGDPAFANYVVSTGIPYRRTVNERDIVPHLPPAAFGFLHAGEEYWIT   319
 35  MDLA_PENCA  SPRVGNAALAKYITAQGNNF-RFTHTNDPVPKLPLLSMGYVHVSPEYWIT   247
                  ****....* *. . *  . * ....* **..*  ..*..* . * **.

     LIP_RHIDL   SGTSN-V-----QICTSEIETKDCSNSIVPFTSILD-HLSYF-DINEGSC   391
     LIP_RHIMI   DNSPETV-----QVCTSDLETSDCSNSIVPFTSVLD-HLSYF-GINTGLC   362
 40  MDLA_PENCA  SPNNATVSTSDIKVIDGDVSFDGNTGTGLPLLTDFEAHIWYFVQVDAGKG   297
                  . . . *     .. ..... .. ... .*. . .. *. **  ...*

     LIP_RHIDL   -------L   392
     LIP_RHIMI   -------T   363
 45  MDLA_PENCA  PGLPFKRV   305
```

EXAMPLE 4

Isolation and purification of *Aspergillus tubigensis* genomic DNA

The *Aspergillus tubigensis* mutant strain was grown in PDB (Difco) for 72 hours and the mycelium was harvested. 0.5-1 g of mycelium was frozen in liquid nitrogen and ground in a mortar. Following evaporation of the nitrogen, the ground mycelium was mixed with 15 ml of an extraction buffer (100 mM Tris·HCl, pH 8.0, 50 mM EDTA, 500 mM NaCl, 10 mM ß-mercaptoethanol) and 1 ml 20 % sodium dodecylsulfate. The mixture was vigorously mixed and incubated at 65°C for 10 min. 5 ml 3M potassium acetate, (pH 5.1 adjusted with glacial acetic acid) was added and the mixture further incubated on ice for 20 min. The cellular debris was removed by centrifugation for 20 min. at 20,000 x g and 10 ml isopropanol was added to the supernatant to precipitate (30 min at -20°C) the extracted DNA. After further centrifugation for 15 min at 20,000 x g, the DNA pellet was dissolved in 1 ml TE (10 mM Tris·HCl pH 8.0, 1 mM EDTA) and precipitated again by addition of 0.1 ml 3 M NaAc, pH 4.8 and 2.5 ml ethanol. After centrifugation for 15 min at 20,000 x g the DNA pellet was washed with 1 ml 70 % ethanol and dried under vacuum. Finally, the DNA was dissolved in 200 µl TE and stored at -20°C.

EXAMPLE 5

The generation of a fragment of the putative gene coding for lipase 3 using PCR

To obtain a fragment of the putative gene (in the following referred to as the *lip*A gene) as a tag to isolate the complete gene, a PCR amplification procedure based on the information in the isolated peptide sequences was carried out.

Degenerated primers for PCR amplification of a fragment of

the lipase gene were designed based on the amino acid sequences of the isolated peptides. The following three PCR primers were synthesised:

C035: TTC CAR AAN CCN GTR TGN AC (SEQ ID NO:4)

20 mer 256 mixture, based on peptide 1 sequence VHTGFWK (Reversed).

C036: CAR YTN TTY GCN CAR TGG (SEQ ID NO:5)

18 mer 256 mixture, based on the N-terminal sequence QLFAQW.

C037: GCV GCH SWY TCC CAV GC (SEQ ID NO:6)

17 mer 216 mixture, based on internal peptide 2 sequence AWESAA (reversed).

The oligonucleotides were synthesised on a Applied Biosystems model 392 DNA/RNA Synthesizer. To reduce the degree of degeneracy the rare Ala codon GCA and the Ser codon TCA have been excluded in design of primer C037.

With these primers the desired fragments were amplified by PCR. Using these primers it was expected that a fragment of about 300 bp should be amplified provided there are no introns in the fragment.

The following PCR reactions were set up in 0.5 ml PCR tubes to amplify a putative *lip*A fragment:

1. 0.5 µg total genomic DNA,
   100 pmol primer C036,
   100 pmol primer C037,
   10 µl PCR Buffer II (Perkin Elmer),
   6 µl 25 mM $MgCl_2$,
   2 µl dNTP mix (10 mM dATP, 10 mM dCTP, 10 mM dGTP, 10 mM dTT
   2 units Amplitaq polymerase (Perkin Elmer), and

water to a total volume of 100 µl.

2. 0.5 µg total genomic DNA,
100 pmol primer C035,
100 pmol primer C036,
10 µl PCR Buffer II (Perkin Elmer),
6 µl 25 mM $MgCl_2$,
2 µl dNTP mix (10 mM dATP, 10 mM dCTP, 10 mM dGTP, 10 mM dTT
2 units Amplitaq polymerase (Perkin Elmer), and
water to a total volume of 100 µl.

The reactions were performed using the following program:

94°C	2 min	
94°C	1 min)	
40°C	1 min)	
72°C	1 min)	These three steps were repeated for 30
72°C	5 min	cycles
5°C	SOAK	

The PCR amplifications were performed in a MJ Research Inc. PTC-100 Thermocycler.

In reaction 1, three distinct bands of about 300, 360 and 400 bp, respectively could be detected. These bands were isolated and cloned using the pT7-Blue-T-vector kit (Novagene). The sizes of these fragment is in agreement with the expected size provided that the fragment contains 0, 1 or 2 introns, respectively.

The three fragments were sequenced using a "Thermo Sekvenase fluorescent labelled primer cycle sequencing Kit" (Amersham) and analyzed on a ALF sequencer (Pharmacia) according to the instructions of the manufacturer. The fragment of about 360 bp contained a sequence that was identified as a lipase and, as it contained the part of the N-terminal distal to the sequence used for primer design, it was concluded that the desired *lip*A gene fragment was obtained.

The sequence of the about 360 bp PCR fragment (SEQ ID NO:7) is shown in the following Table 5.1. The peptide sequence used for primer design is underlined. The remaining part of the N-terminal sequence is doubly underlined.

Table 5.1. PCR-generated putative *lip*A sequence

```
                10        20        30        40        50        60
10              |         |         |         |         |         |
tacccggggntccgattCAGTTGTTCGCGCAATGGTCTGCCGCAGCTTATTGCTCGAATA

                          Q  L  F  A  Q  W  S  A  A  A  Y  C  S  N

                70        80        90       100       110       120
15              |         |         |         |         |         |
ATATCGACTCGAAAGAVTCCAACTTGACATGCACGGCCAACGCCTGTCCATCAGTCGAGG

                N  I  D  S  K  X  S  N  L  T  C  T  A  N  A  C  P  S  V  E
20
                       130       140       150       160       170       180
                        |         |         |         |         |         |
AGGCCAGTACCACGATGGTGCTGGAGTTCGACCTGTATGTCACTCAGATCGCAGACATAG

25              E  A  S  T  T  M  L  L  E  F  D  L  Y  V  T  Q  I  A  D  I

                       190       200       210       220       230       240
                        |         |         |         |         |         |
AGCACAGCTAATTGAACAGGACGAACGACTTTTGGAGGCACAGCCGGTTTCCTGGCCGCG
30
                E  H  S  -  L  N  R  T  N  D  F  W  R  H  S  R  F  P  G  R

                       250       260       270       280       290       300
                        |         |         |         |         |         |
35              GACAACACCAACAAGCGGCTCGTGGTCGCCTTCCGGGGAAGCAGCACGATTGAGAACTGG

                G  Q  H  Q  Q  A  A  R  G  R  L  P  G  K  Q  H  D  -  E  L

                       310       320       330
                        |         |         |
40              ATTGCTAATCYTGACTTCATCCTGGRAGATAACG

                D  C  -  X  -  L  H  P  X  R  -
```

The finding of this sequence permitted full identification of the PCR fragment as part of the *lip*A gene. The stop codon found in the reading frame can be caused either by a PCR or a reading error or there can be an intron encoded in the fragment as a consensus intron start and ending signal (shown in bold). If the putative intron is removed a shift in reading frame will occur. However, an alignment of the deduced amino acid sequence and the fungal lipases shown in Table 3.1 suggested that the fragment was part of the desired gene.

EXAMPLE 6

Cloning and characterisation of the *lip*A gene

(i) Construction of an *Aspergillus tubigensis* genomic library

Aspergillus tubigensis genomic DNA was digested partially with Tsp5091 (New England Biolabs Inc.). 10 µg DNA was digested in 100 µl reaction mixture containing 2 units Tsp5091. After 5, 10, 15 and 20 minutes 25 µl was removed from the reaction mixture and the digestion was stopped by addition of 1 µl 0.5 M EDTA, pH 8.0. After all four reactions had been stopped, the samples were run on a 1% agarose gel in TAE buffer (10 x TAE stock containing per litre: 48.4 g Trizma base, 11.5 ml glacial acetic acid, 20 ml 0.5 M EDTA pH 8.0). *Hin*dIII-digested phage Lambda DNA was used as molecular weight marker (DNA molecular weight marker II, Boehringer, Mannheim). Fragments of a size between about 5 and 10 kb were cut out of the gel and the DNA fragments were purified using Gene Clean II Kit (Bio-101 Inc.). The purified fragments were pooled and 100 ng of the pooled fragments were ligated into 1 µg *Eco*RI-digested and dephosphorylated ZAP II vector (Stratagene) in a total volume of 5 µl. 2 µl of this volume was packed with Gigapack II packing extract (Stratagene) which gave a primary library of 650,000 pfu.

E. coli strain XL1-Blue-MRF (Stratagene) was infected with 5x 50,000 pfu of the primary library. The infected bacteria were

mixed with top agarose (as NZY plates but with 6 g agarose per litre instead of the agar) and plated on 5 NZY plates (13 cm). After incubation at 37°C for 7 hours, 10 ml SM buffer (per litre: 5.8 g NaCl, 2.0 g $MgCl_2 \cdot 7H_2O$, 50 ml 1 M Tris·HCl pH 7.5, 5.0 ml of 2% (w/v) gelatine) and incubated overnight at room temperature with gently shaking. The buffer containing washed-out phages was collected and pooled. 5% chloroform was added and after vigorous mixing the mixture was incubated 1 hour at room temperature. After centrifugation for 2 minutes at 10,000 x g the upper phase containing the amplified library was collected and dimethylsulphoxide was added to 7%. Aliquots of the library was taken out in small tubes and frozen at -80°C. The frozen library contained 2.7×10^9 pfu/ml with about 6% without inserts.

(ii) Screening of the *Aspergillus tubigensis* library

2 x 50.000 pfu were plated on large (22 x 22 cm) NZY plates containing a medium containing per litre: 5 g NaCl, 2 g $MgSO_4 \cdot 7H_2O$, 5 g yeast extract, 10 g casein hydrolysate, 15 g agar, pH adjusted to 7.5 with NaOH. The medium was autoclaved and cooled to about 60°C and poured into the plates. Per plate was used 240 ml of medium.

The inoculated NZY plates were incubated overnight at 37°C and plaque lifts of the plates were made. Two lifts were made for each plate on Hybond N (Amersham) filters. The DNA was fixed using UV radiation for 3 min. and the filters were hybridized as described in the following using, as the probe, the above PCR fragment of about 360 bp that was labelled with ^{32}P-dCTP using Ready-to-Go labelling kit (Pharmacia).

The filters were prehybridised for one hour at 65°C in 25 ml prehybridisation buffer containing 6.25 ml 20 x SSC (0.3 M Na_3citrate, 3 M NaCl), 1,25 ml 100 x Denhard solution, 1.25 ml 10% SDS and 16.25 ml water. 150 µl 10 mg/ml denatured Salmon sperm DNA was added to the prehybridization buffer

immediately before use. Following prehybridization, the prehybridisation buffer was discarded and the filters hybridised overnight at 65°C in 25 ml prehybridisation buffer with the radiolabelled PCR fragment.

Next day the filters were washed according to the following procedure: 2 x 15 min. with 2 x SSC + 0.1 % SDS, 15 min. with 1 x SSC + 0.1 % SDS and 10 min. with 0.1 x SSC + 0.1% SDS.

All washes were done at 65°C. The sheets were autoradiographed for 16 hours and positive clones were isolated. A clone was reckoned as positive only if there was a hybridisation signal on both plaque lifts of the plate in question.

Seven putative clones were isolated and four were purified by plating on small petri dishes and performing plaque lifts essentially as described above.

The purified clones were converted to plasmids using an ExAssist Kit (Stratagene).

Two sequencing primers were designed based on the about 360 bp PCR fragment. The sequencing primers were used to sequence the clones and a positive clone with the *lip*A gene encoding lipase 3 was found. The isolated positive clone was designated pLIP4.

(iii) Characterisation of the pLIP4 clone

A restriction map of the clone was made. The above 360 bp PCR fragment contained a *Sac*II site and as this site could be found in the genomic clone as well this site facilitated the construction of the map. The restriction map showing the structure of pLIP4 is shown in Fig. 1. The restriction map shows that the complete gene is present in the clone. Additionally, since promoter and terminator sequences are present, it was assumed that all the important regions is pre-

sent in the clone.

A sample of *Escherichia coli* strain DH5α containing pLIP4 was deposited in accordance with the Budapest Treaty with The National Collections of Industrial and Marine Bacteria Limited (NCIMB) at 23 St. Machar Drive, Aberdeen, Scotland, United Kingdom, AB2 1RY on 24 February 1997 under the accession number NCIMB 40863.

The gene was sequenced using cycle sequencing and conventional sequencing technology. The complete sequence (SEQ ID NO:8) is shown below in Table 6.1. The sequence has been determined for both strands for the complete coding region and about 100 bp upstream and downstream of the coding region. The sequences downstream to the coding region have only been determined on one strand and contains a few uncertainties. In the sequence as shown below, the intron sequences are indicated as lowercase letters and the N-terminal and the two internal peptides (peptide 1 and peptide 2) are underlined:

Table 6.1. The DNA sequence for the *lip*A gene and flanking sequences

```
1    CCNDTTAATCCCCCACCGGGGTTCCCGCTCCCGGATGGAGATGGGGCCAAAACTGGCAAC
61   CCCCAGTTGCGCAACGGAACAACCGCCGACCCGGAACAAAGGATGCGGATGAGGAGATAC
121  GGTGCCTGATTGCATGGCTGGCTTCATCTGCTATCGTGACAGTGCTCTTTGGGTGAATAT
181  TGTTGTCTGACTTACCCCGCTTCTTGCTTTTTCCCCCCTGAGGCCCTGATGGGGAATCGC
241  GGTGGGTAATATGATATGGGTATAAAAGGGAGATCGGAGGTGCAGTTGGATTGAGGCAGT
301  GTGTGTGTGTGCATTGCAGAAGCCCGTTGGTCGCAAGGTTTTGGTCGCCTCGATTGTTTG
361  TATACCGCAAGATGTTCTCTGGACGGTTTGGAGTGCTTTTGACAGCGCTTGCTGCGCTGG
                M  F  S  G  R  F  G  V  L  L  T  A  L  A  A  L
421  GTGCTGCCGCGCCGGCACCGCTTGCTGTGCGGAgtaggtgtgcccgatgtgagatggttg
     G  A  A  A  P  A  P  L  A  V  R
481  gatagcactgatgaagggtgaatagGTGTCTCGACTTCCACGTTGGATGAGTTGCAATTG
                                S  V  S  T  S  T  L  D  E  L  Q  L
541  TTCGCGCAATGGTCTGCCGCAGCTTATTGCTCGAATAATATCGACTCGAAAGACTCCAAC
     F  A  Q  W  S  A  A  A  Y  C  S  N  N  I  D  S  K  D  S  N
601  TTGACATGCACGGCCAACGCCTGTCCATCAGTCGAGGAGGCCAGTACCACGATGCTGCTG
      L  T  C  T  A  N  A  C  P  S  V  E  E  A  S  T  T  M  L  L
661  GAGTTCGACCTgtatgtcactcagatcgcagacatagagcacagctaatttgaacagGAC
```

```
      E  F  D  L                                                    T
721   GAACGACTTTGGAGGCACAGCCGGTTTCCTGGCCGCGGACAACACCAACAAGCGGCTCGT
        N  D  F  G  G  T  A  G  F  L  A  A  D  N  T  N  K  R  L  V
781   GGTCGCCTTCCGGGGAAGCAGCACGATTGAGAACTGGATTGCTAATCTTGACTTCATCCT
        V  A  F  R  G  S  S  T  I  E  N  W  I  A  N  L  D  F  I  L
841   GGAAGATAACGACGACCTCTGCACCGGCTGCAAGGTCCATACTGGTTTCTGGAAGGCATG
        E  D  N  D  D  L  C  T  G  C  K  V  H  T  G  F  W  K  A  W
901   GGAGTCCGCTGCCGACGAACTGACGAGCAAGATCAAGTCTGCGATGAGCACGTATTCGGG
        E  S  A  A  D  E  L  T  S  K  I  K  S  A  M  S  T  Y  S  G
961   CTATACCCTATACTTCACCGGGCACAGTTTGGGCGGCGCATTGGCTACGCTGGGAGCGAC
        Y  T  L  Y  F  T  G  H  S  L  G  G  A  L  A  T  L  G  A  T
1021  AGTTCTGCGAAATGACGGATATAGCGTTGAGCTGgtgagtccttcacaaaggtgatggag
        V  L  R  N  D  G  Y  S  V  E  L
1081  cgacaatcgggaacagacagtcaatagTACACCTATGGATGTCCTCGAATCGGAAACTAT
                                    Y  T  Y  G  C  P  R  I  G  N  Y
1141  GCGCTGGCTGAGCATATCACCAGTCAGGGATCTGGGGCCAACTTCCGTGTTACACACTTG
        A  L  A  E  H  I  T  S  Q  G  S  G  A  N  F  R  V  T  H  L

1201  AACGACATCGTCCCCCGGGTGCCACCCATGGACTTTGGATTCAGTCAGCCAAGTCCGGAA
        N  D  I  V  P  R  V  P  P  M  D  F  G  F  S  Q  P  S  P  E
1261  TACTGGATCACCAGTGGCAATGGAGCCAGTGTCACGGCGTCGGATATCGAAGTCATCGAG
        Y  W  I  T  S  G  N  G  A  S  V  T  A  S  D  I  E  V  I  E
1321  GGAATCAATTCAACGGCGGGAAATGCAGGCGAAGCAACGGTGAGCGTTGTGGCTCACTTG
        G  I  N  S  T  A  G  N  A  G  E  A  T  V  S  V  V  A  H  L
1381  TGGTACTTTTTTGCGATTTCCGAGTGCCTGCTATAACTAGACCGACTGTCAGATTAGTGG
        W  Y  F  F  A  I  S  E  C  L  L  -
1441  ACGGGAGAAGTGTACATAAGTAATTAGTATATAATCAGAGCAACCCAGTGGTGGTGATGG
1501  TGGTGAAAGAAGAAACACATTGAGTTCCCATTACGKAGCAGWTAAAGCACKTKKGGAGGC
1561  GCTGGTTCCTCCACTTGGCAGTTGGCGGCCATCAATCATCTTTCCTCTCCTTACTTTCGT
1621  CCACCACAACTCCCATCCTGCCAGCTGTCGCATCCCCGGGTTGCAACAACTATCGCCTCC
1681  GGGGCCTCCGTGGTTCTCCTATATTATTCCATCCGACGGCCGACGTTTCACCCTCAACCT
1741  GCGCCGCCGCAAAATCTCCCCGAGTCGGTCAACTCCCTCGAACCGCCGCCCGCATCGACC
1801  TCACGACCCCGACCGTCTGYGATYGTCCAACCG
```

(iv) Analysis of the sequence of the complete gene

The peptide sequences obtained could all be found in the deduced amino acid sequence (see Table 5.1) which confirms again that the sequence found is the sequence of the lipase 3 gene. The gene was designated *lip*A.

The amino acid sequence was aligned with the three fungal lipases used to align the peptide sequences. The alignment is shown in Table 6.2.

Table 6.2 Alignment of the lipase 3 sequence with known fungal lipases

```
 5 LIPASE3     MFSG-----------RFGVLL-----------------------TALAA    15
   MDLA_PENCA  MRLS-----------FFTAL-----------------------SAVAS    14
   LIP_RHIDL   MVSFISISQGVSLCLLVSSMMLGSSAVPVSGKSGSSNTAVSASDNAALPP   50
   LIP_RHIMI   MVLKQRANYLGFLIVFFTAFLV--EAVPIKRQSNSTVDS--------LPP   40
               *                . .                          ...
10
   LIPASE3     L-------------------------------------------------   16
   MDLA_PENCA  L-------------------------------------------------   15
   LIP_RHIDL   LISSRCAPPSNKGSKSDLQAEPYNMQKNTEWYESHGGNLTSIGKRDDNLV  100
   LIP_RHIMI   LIPSRTSAPSSSPSTTDPEAPAM----------SRNGPLPS----DVETK   76
15             *

   LIPASE3     --------GAAAPAPLA-----------VRSVSTSTLDELQLFAQWSAAA   47
   MDLA_PENCA  --------GYALPGKLQ-----------SRDVSTSELDQFEFWVQYAAAS   46
   LIP_RHIDL   GGMTLDLPSDAPPISLSSSTNSASDGGKVVAATTAQIQEFTKYAGIAATA  150
20 LIP_RHIMI   YGMALNATSYPDSV-----VQAMSIDGGIRAATSQEINELTYYTTLSANS  121
                        . . .                 ....  ....  ..  .*..

   LIPASE3     YCSNNIDSK-DSNLTCTANACPSVEEASTTMLLEFDLTNDFGGTAGFLAA   96
   MDLA_PENCA  YYEADYTAQVGDKLSCSKGNCPEVEATGATVSYDFS-DSTITDTAGYIAV   95
25 LIP_RHIDL   YCRSVVP---GNKWDCVQ--CQKWVPDGKIIT---TFTSLLSDTNGYVLR  192
   LIP_RHIMI   YCRTVIP---GATWDCIH--CDA-TEDLKIIK---TWSTLIYDTNAMVAR  162
               *. .      ... .*    *     . ..    . .. . .*.. .

   LIPASE3     DNTNKRLVVAFRGSSTIENWIANLDFILEDNDDLCTGCKVHTGFWKAWES  146
30 MDLA_PENCA  DHTNSAVVLAFRGSYSVRNWVADATFV-HTNPGLCDGCLAELGFWSSWKL  144
   LIP_RHIDL   SDKQKTIYLVFRGTNSFRSAITDIVFNFSDYKPV-KGAKVHAGFLSSYEQ  241
   LIP_RHIMI   GDSEKTIYIVFRGSSSIRNWIADLTFVPVSYPPV-SGTKVHKGFLDSYGE  211
               ..... . ..***. .  . ...  *   .   . .*  .. ** ...

35 LIPASE3     AADELTSKIKSAMSTYSGYTLYFTGHSLGGALATLGATVL--RNDGY-SV  193
   MDLA_PENCA  VRDDIIKELKEVVAQNPNYELVVVGHSLGAAVATLAATDL--RGKGYPSA  192
   LIP_RHIDL   VVNDYFPVVQEQLTAHPTYKVIVTGHSLGGAQALLAGMDLYQREPRLSPK  291
   LIP_RHIMI   VQNELVATVLDQFKQYPSYKVAVTGHSLGGATALLCALDLYQREEGLSSS  261
               . ..    . .     ..*..  .*****.* * * .  *  *.    .
40
   LIPASE3     ELYTY--GCPRIGNYALAEHITSQGSGANFRVTHLNDIVPRVPPMDFGFS  241
   MDLA_PENCA  KLYAY--ASPRVGNAALAKYITAQGN--NFRFTHTNDPVPKLPLLSMGYV  238
   LIP_RHIDL   NLSIFTVGGPRVGNPTFAYYVESTGIPFQ-RTVHKRDIVPHVPPQSFGFL  340
   LIP_RHIMI   NLFLYTQGQPRVGDPAFANYVVSTGIPYR-RTVNERDIVPHLPPAAFGFL  310
45             .*  .  . **.*. ..* .. . *   . * .. .* **..*  ..*.
```

```
LIPASE3     QPSPEYWITSGNGASVTASDIEVIEGINSTAGNAGEATVSVV---AHLWY   288
MDLA_PENCA  HVSPEYWITSPNNATVSTSDIKVIDGDVSFDGNTGTGLPLLTDFEAHIWY   288
LIP_RHIDL   HPGVESWIKSGTSN-VQICTSEIE------TKDCSNSIVPFTSILDHLSY   383
LIP_RHIMI   HAGEEYWITDNSPETVQVCTSDLE------TSDCSNSIVPFTSVLDHLSY   354
            . . * **. . . * .. ..      . . ...    .   .*. *

LIPASE3     FFAISECL--------L   297
MDLA_PENCA  FVQVDAGKGPGLAFKRV   305
LIP_RHIDL   F-DINEGSC-------L   392
LIP_RHIMI   F-GINTGLC------T    363
            *  ...
```

The above alignment shows that lipase 3 is homologous to the known lipase sequences but that the homology is not very high. Deletions or insertions in the lipase 3 sequence was not observed when comparing the sequence with these three lipases. This strengthens the probability that the putative introns have been identified correctly.

A search in SWISS-PROT release 31 database was performed and it did not lead to further sequences with higher homology than that to the above known lipases (Table 6.3).

The sequence with highest homology is a mono- diacyl lipase from *Penicillium camembertii* where the identity is found to 42 %. However the C- terminal of lipase 3 resembles the 2 lipases from Zygomycetes (*Rhizopus* and *Rhizomucor*) and not the *P. camembertii* enzyme.

Table 6.3. Alignment of coding sequences of the *lip*A gene and gene coding for mono-diacyl lipase from *Penicillium camemberti*

```
LIPASE3    - MFSGRFGVLLTALAALGAAAPAPLAVRSVSTSTLDELQLFAQWSAAAYCS -50
MDLA_PENCA- MRLSFFTAL-SAVASLGYALPGKLQSRDVSTSELDQFEFWVQYAAASYYE -49

LIPASE3    - NNIDSK-DSNLTCTANACPSVEEASTTMLLEFDLTNDFGGTAGFLAADNT -99
MDLA_PENCA- ADYTAQVGDKLSCSKGNCPEVEATGATVSYDFS-DSTITDTAGYIAVDHT -98
```

```
LIPASE3    - NKRLVVAFRGSSTIENWIANLDFILEDNDDLCTGCKVHTGFWKAWESAAD -149
                 |   | |||||     || |   |    |  || ||    |||  |    |
MDLA_PENCA- NSAVVLAFRGSYSVRNWVADATFV-HTNPGLCDGCLAELGFWSSWKLVRD -147

LIPASE3    - ELTSKIKSAMSTYSGYTLYFTGHSLGGALATLGATVLRNDGY-SVELYTY -198
                    |        | |   ||||| | ||| || ||  || |  || |
MDLA_PENCA- DIIKELKEVVAQNPNYELVVVGHSLGAAVATLAATDLRGKGYPSAKLYAY -197

LIPASE3    - GCPRIGNYALAEHITSQGSGANFRVTHLNDIVPRVPPMDFGFSQPSPEYW -248
               || || |||  || ||   ||| || || ||  |    |    |||||
MDLA_PENCA- ASPRVGNAALAKYITAQGN--NFRFTHTNDPVPKLPLLSMGYVHVSPEYW -245

LIPASE3    - ITSGNGASVTASDIEVIEGINSTAGNAGEATVSVV---AHLWYFFAISEC -295
                |||  | | |  |||  || |  |  || |          || |||
MDLA_PENCA- ITSPNNATVSTSDIKVIDGDVSFDGNTGTGLPLLTDFEAHIWYFVQVDAG -295

LIPASE3    - L--------L -297

MDLA_PENCA- KGPGLPFKRV -305

Identity: 126 amino acids (42.42%)
```

The N-terminal of the mature lipase has been determined by N-terminal sequencing to be the serine residue No. 28 of the lipase 3 precursor (SEQ ID NO:9) as shown in Table 6.4 below. Hence the amino acids No. 1 to No. 27 is the signal sequence.

Table 6.4: Amino acid sequence of the precursor of lipase 3

```
                  5         10        15        20        25        30
                  |         |         |         |         |         |
    1 M F S G R F G V L L T A L A A L G A A A P A P L A V R S V S
   31 T S T L D E L Q L F A Q W S A A A Y C S N N I D S K D S N L
   61 T C T A N A C P S V E E A S T T M L L E F D L T N D F G G T
   91 A G F L A A D N T N K R L V V A F R G S S T I E N W I A N L
  121 D F I L E D N D D L C T G C K V H T G F W K A W E S A A D E
  151 L T S K I K S A M S T Y S G Y T L Y F T G H S L G G A L A T
  181 L G A T V L R N D G Y S V E L Y T Y G C P R I G N Y A L A E
  211 H I T S Q G S G A N F R V T H L N D I V P R V P P M D F G F
  241 S Q P S P E Y W I T S G N G A S V T A S D I E V I E G I N S
  271 T A G N A G E A T V S V V A H L W Y F F A I S E C L L
```

Number of residues : 297.

Residues 167-176 are recognised as a common motif for the serine lipases (PROSITE). The crystal structure for the *Rhizomucor miehei* serine lipase has been examined and the residues in the active site identified (Brady et al., Nature, 1990, **343**:767-770; Derewanda et al., J. Mol. Biol., 1992, **227**:818-839). The active site residues of *R. miehei* lipase have all been conserved in all the lipases and correspond to the following residues in lipase 3: serine 173, aspartic acid 228 and histidine 285.

Lipase 3 contains 7 cysteine residues. Four of these are conserved in the *P. camembertii* lipase where they form disulphide bonds (Isobe and Nokuhara, Gene, 1991, **103**:61-67). This corresponds to disulphide bonds between residue 62- 67 and 131-134. In addition, two cysteine residues are homologous to two C residues which forms an additional disulphide bond in *Rhizopus* and *Rhizomucor* lipases corresponding to residues 49-295.

Two putative N-glycosylation sites were found in lipase 3 in position 59 and 269. Neither of these are conserved in the other fungal lipases.

EXAMPLE 7

Transformation of *Aspergillus tubigensis* and overexpression of lipase 3 in *A. tubigensis*

The protocol for transformation was based on the teachings of Buxton et al. (Gene, 1985, **37**:207-214), Daboussi et al (Curr. Genet., 1989, **15**:453-456) and Punt and van den Hondel, (Meth. Enzym., 1992, **216**:447-457).

A multicopy *lip*A strain was produced by transforming the pLIP4 plasmid into *Aspergillus tubigensis* strain 6M 179 using cotransformation with a hygromycin resistant marker plasmid.

A screening procedure used to visualise fungal lipase after ultrathin layer isoelectric focusing was adapted to screen *Aspergillus tubigensis* transformants grown on agar plates. Screening of lipase producers on agar plates was done using 2% olive oil as the substrate for the enzyme (lipase) as well as the inducer for the lipase promoter. In addition, the plates contained a fluorescent dye, Rhodamine B. In the presence of olive oil, the transformants will be induced to secrete lipase. The lipase secreted into the agar plate will hydrolyse the olive oil causing the formation of orange fluorescent colonies that is visible upon UV radiation (350 nm). The appearence of fluorescent colonies was generally monitored after 24 hours of growth. After several days of growth, the lipase producing strains could be identified as orange fluorescent strains that are visible by eye. Under this plate screening condition, the untransformed strain gave no background fluorescence and appeared as opaque pink colonies.

Sixteen transformants that showed orange fluorescent halos were cultivated for 8 days in shake flasks containing 100 ml of minimal medium supplemented with 1% olive oil, 0.5% yeast extract and 0.2% casamino acids. The amount of lipase secreted was quantified by applying 10 µl of cell-free culture supernatant into holes punched in olive oil- Rhodamine B agar plates and incubating the plates overnight at 37°C. Five transformants with higher lipase production were found.

The cell-free culture supernatants from the five transformants were desalted using NAP 5 columns (Pharmacia) and equilibrated in 1M ammonium sulfate (50 mM sodium acetate, pH 5.5). The desalted culture supernatants were fractionated by hydrophobic interaction chromatography (HIC) on a Biogel Phenyl-5 PW column (Biorad). Elution was done by a descending salt gradient of 1M to 0 M ammonium sulfate (20 mM sodium acetate, pH 5.5). A single discrete protein peak was observed after fractionation. The area of the protein peaks were calculated among the different transformants and compared

with the untransformed strain. The best transformant showed a 62-fold increase in the amount of lipase after HIC fractionation. A chromatogram of the HIC fractionated culture supernatant of this transformant is shown in Fig. 3 and a similar chromatogram for the untransformed strain is shown in Fig. 4.

The fraction containing the transformed lipase was freeze-dried. The transformed lipase was carboxymethylated and subjected to N-terminal amino acid sequencing of the first 15 amino acids and it was found that the sequence of the recombinant lipase was exactly the same as the native lipase indicating correct signal sequence cleavage.

The different lipase fractions collected after HIC were separated on a 12% Tris-Glycine SDS gel and silver staining revealed one protein band, confirming the homogeneity of the fractions. In addition, the crude extract showed a major lipase band as the only band that accumulated in the culture supernatant in very high amounts when the fungus was cultured in the olive oil-containing medium.

The recombinant lipase was analysed by matrix-assisted laser desorption ionisation (MALDI) by means of a time-of-flight (TOF) mass spectrometer as described hereinbefore. The molecular weight of the recombinant lipase was 32,237 Da.

Detection of N-linked oligosaccharides was achieved by digestion of the lipase with endo-β-N-acetyl-glucosamidase H from *Streptomyces* (Sigma). Digestion of recombinant lipase secreted into the growth medium altered the mobility of the band seen on SDS-PAGE which moved as a single band with a molecular mass of about 30 kDa.

Deglycosylated recombinant lipase generated by digestion with endoglycosidase and analysed directly by MALDI mass spectrometry gave a molecular weight of the polypeptide backbone of 29,325 Da.

C. BAKING EXPERIMENTS

EXAMPLE 8

Baking experiments using lipase 3

8.1. Baking procedures and analytical methods

(i) Baking procedure for Danish toast bread

Flour (Danish reform flour) 2000 g, dry yeast 30 g, salt 30 g and water corresponding to 400 Brabender units + 3%, was kneaded in a Hobart Mixer with hook for 2 min. at low speed and 10 min. at high speed. Dough temperature after kneading was 25°C. Resting time was 10 min. at 30°C. The dough was scaled 750 g per dough and rested again for 5 min at 33°C and 85% RH. After moulding on a Glimik moulder, the dough were proofed in tins for 50 min at 33°C and baked in a Wachtel oven for 40 min at 220°C with steam injection for 16 sec. After cooling, the bread was scaled and the volume of the bread was measured by the rape seed displacement method. The specific volume is calculated by dividing the bread volume (ml) by the weight (g) of the bread.

The crumb was evaluated subjectively using a scale from 1 to 5 where 1 = coarsely inhomogeneous and 5 = nicely homogeneous.

Three breads baked in tins with lid were stored at 20°C and used for firmness measurements and pore measurements by means of an Image Analyzer.

(ii) Baking procedure for Danish rolls

Flour (Danish reform) 1500 g, compressed yeast 90 g, sugar 24 g, salt 24 g and water corresponding to 400 Brabender units - 2% were kneaded in a Hobart mixer with hook for 2 min. at low

speed and 9 min at high speed. After kneading, the dough temperature was 26°C. The dough was scaled 1350 g. After resting for 10 min. at 30°C, the dough was moulded on a Fortuna moulder after which the dough was proofed for 45 min. at 34°C and baked in a Bago oven for 18 min. at 220°C with steam injection for 12 sec. After cooling, the rolls were scaled and the volume of the rolls was measured by the rape seed displacement method. Specific volume is calculated as described above.

(iii) Determination of pore homogeneity

The pore homogeneity of the bread was measured by means of an image analyzer composed of a standard CCD-video camera, a video digitiser and a personal computer with WinGrain software. For every bread, the results of pore diameter in mm and pore homogeneity were calculated as an average of measurements from 10 slices of bread. The pore homogeneity was expressed in % of pores that are larger than 0.5 times the average of pore diameter and smaller than 2 times the average diameter.

(iv) Determination of firmness

The firmness of bread, expressed as N/dm^2, was measured by means of an Instron UTM model 4301 connected to a personal computer. The conditions for measurement of bread firmness were:

Load Cell	Max. 100 N
Piston diameter	50 mm
Cross head speed	200 mm/min
Compression	25%
Thickness of bread slice	11 mm

The result was an average of measurements on 10 bread slices for every bread.

(v) Determination of gluten index

Gluten index was measured by means of a Glutomatic 2200 from Perten Instruments (Sweden). Immediately after proofing, 15 g of dough was scaled and placed in the Glutomatic and washed with 500 ml 2% NaCl solution for 10 min. The washed dough was transferred to a Gluten Index Centrifuge 2015 and the two gluten fractions were scaled and the gluten index calculated according to the following equation:

Gluten index = (weight of gluten remaining on the sieve x 100)/total weight of gluten

(vi) Extraction of lipids from dough

30 g of fully proofed dough was immediately frozen and freeze-dried. The freeze-dried dough was milled in a coffee mill and passed through a 235 µm screen. 4 g freeze-dried dough was scaled in a 50 ml centrifuge tube with screw lid and 20 ml water saturated n-butanol (WSB) was added. The centrifuge tube was placed in a water bath at a temperature of 100°C for 10 min. after which the tubes were placed in a Rotamix and turned at 45 rpm for 20 min. at ambient temperature. The tubes were again placed in the water bath for 10 min. and turned on the Rotamix for another 30 min. at ambient temperature.

The tubes were centrifuged at 10,000 x g for 5 min. 10 ml of the supernatant was pipetted into a vial and evaporated to dryness under nitrogen cover. This sample was used for HPLC analysis.

A similar sample was fractionated on a Bond Elut Si (Varian 1211-3036). The non-polar fraction was eluted with 10 ml cyclohexan:isopropanol:acetic acid (55:45:1) and evaporated to dryness. This sample was used for GLC analysis.

(vii) HPLC analysis

Column: LiChrospher 100 DIOL 5 µm (Merck art. 16152) 250x4 mm with a water jacket of a temperature of 50°C.

Mobile phases:
A: heptan:isopropanol:n-butanol:tetrahydrofuran:isooctan:water (64.5:17.5:7:5:5:1)

B: isopropanol:n-butanol:tetrahydrofuran:isooctan:water (73:7:5:5:10)

The mobile phases contained 1 mmol trifluoroacetic acid per 1 mobile phase and were adjusted to pH 6.6 with ammonia.

Pump: Waters 510 equipped with a gradient controller.

Gradient:

Flow (ml/min)	Time (min)	A (%)	B (%)
1.0	0	100	0
1.0	25	0	100
1.0	30	0	100
1.0	35	100	0
1.0	40	100	0

Detector: CUNOW DDL21 (evaporative light-scattering); temperature 100°C; voltage: 600 volt; air flow: 6.0 l/min.

Injector: Hewlett Packard 1050; injection volume: 50 µl.

The samples for analysis were dissolved in 5 ml chloroform:methanol (75:25), sonicated for 10 min and filtered through a 0.45 µm filter.

(viii) GLC analysis

Perkin Elmer 8420 Capillary Gas Chromatograph equipped with WCOT fused silica column 12.5 m x 0.25 mm coated with 0.1 µm

stationary phase of 5% phenyl-methyl-silicone (CP Sil 8 CB from Crompack).
Carrier: Helium
Injection: 1.5 µl with split
Detector: FID 385°C

Oven program:	1	2	3	4
Oven temperature, °C	80	200	240	360
Isothermal time, min	2	0	0	10
Temperature rate, °C/min	20	10	12	--

Sample preparation: 50 mg non-polar fraction of wheat lipids was dissolved in 12 ml heptane:pyridine (2:1) containing 2 mg/ml heptadecane as internal standard. 500 µl of the solution was transferred to a crimp vial and 100 µl N-methyl-N-trimethylsilyl-trifluoracetamide was added. The mixture was allowed to react for 15 min at 90°C.

Calculation: Response factors for mono-, di- and triglycerides and free fatty acids were determined from reference mixtures of these components. Based on these response factors, the glycerides and the free fatty acids were calculated in wheat lipids.

8.2. Baking experiments with lipase 3 in Danish toast bread

The effect of adding lipase 3 to a dough for making Danish toast bread was evaluated. The enzyme was added as a freeze-dried preparation on maltodextrin together with the other ingredients. The results of the baking tests are shown in Tables 8.1 to 8.4.

Table 8.1

Lipase LUS/kg flour	0	5,000	15,000	25,000
Specific volume of bread	4.43	4.43	4.22	4.37
Firmness Day 1	35	33	32	30
Firmness Day 7	90	90	85	73

Table 8.2

Lipase LUS/kg flour	0	5,000	15,000	25,000
Average diameter of the crumb pore, mm	2.96	2.33	2.47	2.65
Homogeneity of crumb pore, %	64.9	73.8	66.0	67.1
Porosity, %	85.9	84.7	85.5	85.1
Gluten index, %	42	45.5	55	65

Table 8.3

Lipase LUS/kg flour	0	5,000	15,000	25,000
Fatty acids, %	0.090	0.148	0.218	0.241
Monoglycerides, %	0.017	0.031	0.035	0.039
Diglycerides, %	0.020	0.036	0.040	0.045
Triglycerides, %	0.790	0.714	0.673	0.622

Table 8.4

Lipase LUS/kg flour	0	5,000	15,000	25,000
Monogalactosyl Diglyceride, %	0.073	0.040	0.025	0.018
Digalactosyl Diglyceride, %	0.244	0.220	0.182	0.127
Digalactosyl Monoglyceride, %	0.008	0.022	0.044	0.054
Phosphatidyl choline, %	0.064	0.073	0.055	0.041
Lysophosphatidyl choline, %	0.164	0.182	0.171	0.165

By the addition of up to about 5,000 LUS/kg flour of the lipase no change in bread volume was observed, but at a higher dosage of lipase 3 there was a tendency to a small but not statistically significant decrease in volume (Table 8.1).

From the results in Table 8.2 it appears that lipase 3 improved the bread crumb homogeneity and that the average diameter of the crumb pores was reduced significantly. The gluten index also clearly correlated to the addition of lipase 3 as an indication of a more firm gluten caused by the modification of the wheat lipid components causing better dough stability and a more homogeneous bread pore structure. However, these modifications appeared to be optimal at the addition of 5,000 LUS/kg flour of lipase 3 whereas a higher dosage resulted in a too strong modification of the wheat gluten.

The results of the GLC and HPLC analyses (Table 8.3) clearly demonstrated that the triglycerides in the dough were hydrolysed. But more interestingly, there was also observed a modification of the glycolipids, monogalactosyl diglyceride

and digalactosyl diglyceride. These components were converted to the more polar components monogalactosyl monoglyceride and digalactosyl monoglyceride. As digalactosyl monoglyceride is a more surface active component than digalactosyl diglyceride it is assumed that this component contributed to the observed improved crumb cell structure and homogeneity. It also appeared that phospholipids like phosphatidyl choline were only modified to a very small extent.

8.3. Baking experiments with lipase 3 in Danish rolls

The effect of adding lipase 3 to a dough for making Danish rolls was evaluated. The enzyme was added as a freeze-dried preparation on maltodextrin together with the other ingredients. The results of the baking tests are shown in Tables 8.5 to 8.7.

Table 8.5

Lipase 3 LUS/kg flour	0	10,000	20,000	30,000
Specific volume of bread (45 min fermentation)	6.86	7.04	6.35	6.36
Specific volume of bread (65 min fermentation)	8.30	8.59	8.23	8.04
Subjective evaluation of crumb homogeneity	3	5	4	4

Table 8.6

Lipase 3 LUS/kg flour	0	10,000	20,000	30,000
Free fatty acids, %	0.060	0.126	0.173	0.211
Monoglycerides, %	0.028	0.050	0.054	0.063
Diglycerides, %	0.103	0.095	0.110	0.104
Triglycerides, %	0.705	0.561	0.472	0.436

Table 8.7

Lipase 3 LUS/kg flour	0	5,000	15,000	25,000
Digalactosyl Diglyceride, %	0.204	0.187	0.154	0.110
Digalactosyl Monoglyceride, %	0.007	0.026	0.047	0.074
Phosphatidyl choline, %	0.077	0.078	0.077	0.063
Lysophosphatidyl choline, %	0.153	0.161	0.162	0.150

It is apparent from the results shown in Table 8.5 that the addition of lipase 3 does not significantly increase the volume of the rolls. Furthermore, lipase 3 was found to improve the homogeneity of the crumb.

The GLC and HPLC analyses of the wheat lipids, as shown in Tables 8.6 and 8.7, demonstrated the modification of these lipids.

EXAMPLE 9

Dough improving effect of glycerol oxidase and lipase

The effect of glycerol oxidase and lipase (separately or in combination) on dough strength was studied in a dough prepared according to the AACC Method 54-10. The dough was subjected to extensiograph measurements (Barbender Extensiograph EXEK/6) also according to AACC Method 54-10 with and with out the addition of glycerol oxidase from *Aspergillus japonicus* combined with lipase from *Aspergillus oryzae* (GRINDAMYL™ EXEL 16, Bakery Enzyme, Danisco Ingredients). The dough with out addition of enzymes served as a control.

The principle of the above method is that the dough after forming is subjected to a load-extension test after resting at 30°C for 45, 90 and 135 minutes, respectively, using an extensigraph capable of recording a load-extension curve (extensigram) which is an indication of the doughs resistance to physical deformation when stretched. From this curve, the resistance to extension, B (height of curve) and the extensibility, C (total length of curve) can be calculated. The B/C ratio (D) is an indication of the baking strength of the flour dough. The results of the experiment are summarized in Table 9.1 below.

Table 9.1: Extensigraph measurements of dough supplemented with glycerol oxidase and lipase

Sample (per kg flour)	Resting time (min)	B-value	C-value	D=B/C
Control	45	220	192	1.15
500 LUS lipase	45	225	190	1.18
1000 U glycerol oxidase	45	300	195	1.54
500 LUS lipase + 1000 U Glycerol oxidase	45	350	198	1.77
Control	90	240	196	1.22
500 LUS lipase	90	245	195	1.16
1000 U Glycerol oxidase	90	330	190	1.74
500 LUS lipase + 1000 U Glycerol oxidase	90	380	192	1.98
Control	135	260	188	1.38
500 LUS lipase	135	265	190	1.39
1000 U Glycerol oxidase	135	380	188	2.02
500 LUS lipase + 1000 U Glycerol oxidase	135	410	190	2.15

When the results from the above experiments are compared with regard to the differences between the control dough and the glycerol oxidase supplemented dough it appears that glycerol oxidase clearly has a strengthening effect. The B/C ratio was increased by 34%, 43% and 46% after 45, 90 and 135 minutes of resting time respectively.

The addition of lipase only did not have any effect on the B/C ratio.

However, when supplementing the dough with a combination of glycerol oxidase and lipase, a further increase in the B/C ratio was seen as compared to bread prepared from dough supplemented with glycerol oxidase only. The B/C ratio was increased by 54%, 62% and 56% after 45, 90 and 135 minutes respectively. This clearly indicates that the combined use of these two enzymes in the preparation of bread products has an enhancing effect on the baking strength.

EXAMPLE 10

Improvement of the specific volume of bread prepared from dough supplemented with glycerol oxidase and lipase

The effect of using glycerol oxidase and lipase (separately or in combination) on the specific bread volume and the crumb homogeneity was tested in a baking procedure for Danish rolls with a dough prepared as described in example 8. Glycerol oxidase from *Aspergillus japonicus* and lipase 3 from *Aspergillus tubigensis* was added to the dough in different amounts. Dough without the addition of enzymes served as control. The fully proofed dough was baked at 220°C for 18 minutes with 12 seconds steam in a Bago-oven. After cooling the rolls were weighed and the volume of the rolls were measured by the rape seed displacement method. The specific bread volume was determined as the volume of the bread (ml) divided by the weight of the bread (g). The crumb homogeneity was evaluated subjectively on a scale from 1 to 7, where 1 = course inhomogeneous and 7 = nice homogeneous.
The results from this experiment are summarized in Table 10.1 below.

Table 10.1: Specific volume and crumb homogeneity in bread supplemented with lipase and glycerol oxidase

Sample (per kg flour)	Specific volume (ml/g)	Crumb homogeneity
Control	5.45	1
1,000 U glycerol oxidase	6.75	2
10,000 LUS lipase	5.65	4
10,000 LUS lipase + 1,000 U glycerol oxidase	7.25	7

As can be seen in the above Table 10.1, the use of glycerol oxidase in the preparing of bread, significantly increased the bread volume (24%) as compared to bread prepared from a similar dough not supplemented with this enzyme. Addition of glycerol oxidase did not improve the crumb homogeneity significantly.

The use of lipase in the preparing of bread did not increase the specific volume of the bread, however a highly increased pore homogeneity was observed.

The combined use of glycerol oxidase and lipase increased the specific volume of the bread with 33% as compared to bread prepared from a similar dough not supplemented with any of the two enzymes.

In addition, the crumb homogeneity was highly improved by the combined use of lipase and glycerol oxidase as compared to the control bread and the breads prepared from dough supplemented with lipase and glycerol oxidase respectively.

This clearly indicates that the combination of lipase and glycerol oxidase in the preparation of bread has a synergis-

tic effect and significantly enhances the shape and appearance of the finished bread product.

EXAMPLE 11

Hydrolysis of triglycerides and formation of glycerol in dough supplemented with lipase

In order to study the hydrolysis of triglycerides and the formation of glycerol in a proofed dough supplemented with lipase, a dough for Danish rolls was prepared in the same manner as described in example 8. Different amounts of lipase (GRINDAMYL™ EXEL 16) was added to the dough, and the total lipid from the fully proofed dough was extracted and analyzed by gas chromatography as described above.

Table 11.1: Triglycerides and glycerol in a dough as a function of lipase addition

Lipase addition (GRINDAMYL™ EXEL 16) (LUS per kg flour)	Glycerol (‰)	Triglycerides (‰)
0	2.2	7.88
500	2.2	6.22
1,250	2.4	5.99
2,500	2.8	5.37
3,750	2.9	5.47
5,000	3.0	5.55
7,500	3.1	5.03
10,000	3.0	4.39

From the above experiment it is clear that the addition of

lipase to a dough has a hydrolyzing effect on the triglycerides present in the dough, which is seen as a decrease in the triglyceride content as function of the increased lipase addition. The resulting level of glycerol increases as a function of the lipase addition.

These results suggests, that the improvement of the B/C ratio and the specific bread volume in bread prepared from dough supplemented with both glycerol oxidase and lipase, as was seen in example 9 and 10, could be due to that lipase addition to a dough is generating glycerol which further can act as substrate for glycerol oxidase.

SUMMARY PARAGRAPHS

The present invention is defined in the claims and the accompanying description.

For convenience other aspects of the present invention are presented herein by way of numbered paragraphs.

1. A method of improving the rheological properties of a flour dough and the quality of the finished product made from the dough, comprising adding to the dough 10 to 10,000 units of a glycerol oxidase per kg of flour.

2. A method according to paragraph 1 wherein the glycerol oxidase is derived from an organism selected from the group consisting of a bacterial species, a fungal species, a yeast species, an animal cell and a plant cell.

3. A method according to paragraph 2 wherein the fungal species is selected from the group consisting of an *Aspergillus* species, a *Neurospora* species and a *Penicillium* species.

4. A method according to paragraph 1 wherein the resistance to extension of the dough in terms of the ratio between resistance to extension (height of curve, B) and the extensibility (length of curve, C), i.e. the B/C ratio, as measured by the AACC method 54-10 is increased by at least 10% relative to that of an otherwise similar dough not containing glycerol oxidase.

5. A method according to paragraph 1 wherein the finished product is selected from the group consisting of a bread product, a noodle product and an alimentary paste product.

6. A method according to paragraph 1 where at least one further enzyme is added to the dough ingredients, dough additives or the dough.

7. A method according to paragraph 6 wherein the further enzyme is selected from the group consisting of a cellulase, a hemicellulase, a starch degrading enzyme, an oxidoreductase, a lipase and a protease.

8. A method of improving the rheological properties of a flour dough and the quality of the finished product made from the dough, comprising adding to the dough a glycerol oxidase and a lipase.

9. A method according to paragraph 8 wherein the amount of glycerol oxidase is in the range of 10 to 10,000 units per kg flour.

10. A method according to paragraph 8 wherein the glycerol oxidase is derived from an organism selected from the group consisting of a bacterial species, a fungal species, a yeast species, an animal cell and a plant cell.

11. A method according to paragraph 10 wherein the fungal species is selected from the group consisting of an *Aspergillus* species, a *Neurospora* species and a *Penicillium* species.

12. A method according to paragraph 8 wherein the resistance to extension of the dough in terms of the ratio between resistance to extension (height of curve, B) and the extensibility (length of curve, C), i.e. the B/C ratio, as measured by the AACC method 54-10 is increased by at least 10% relative to that of an otherwise similar dough not containing glycerol oxidase.

13. A method according to paragraph 8 wherein the finished product is selected from the group consisting of a bread product, a noodle product and an alimentary paste product.

14. A method according to paragraph 8 where at least one

further enzyme is added to the dough ingredients, dough additives or the dough.

15. A method according to paragraph 14 wherein the further enzyme is selected from the group consisting of a cellulase, a hemicellulase, a starch degrading enzyme, an oxidoreductase, and a protease.

16. A method according to paragraph 8 wherein the amount of lipase is in the range of 10 to 100,000 LUS per kg of flour.

17. A method according to paragraph 8 wherein the lipase is derived from an organism selected from the group consisting of a bacterial species, a fungal species, a yeast species, an animal cell and a plant cell.

18. A method according to paragraph 17 wherein the lipase is derived from an *Aspergillus* species.

19. A method according to paragraph 18 wherein the *Aspergillus* species is selected from the group consisting of *A. tubigensis*, *A. oryzae* and *A. niger*.

20. A method according to paragraph 8 wherein at least 10% of the galactosyl diglycerides normally present in a flour dough is hydrolysed to the corresponding galactosyl monoglycerides.

21. A dough improving composition comprising a glycerol oxidase and at least one further dough ingredient or dough additive.

22. A composition according to paragraph 21 wherein the further dough additive is selected from the group consisting of a substrate for glycerol oxidase and a lipase.

23. A composition according to paragraph 22 which is a pre-mixture useful for preparing a baked product or in making a noodle product or an alimentary paste product.

24. A composition according to paragraph 21 which comprises an additive selected from the group consisting of an emulsifying agent and a hydrocolloid.

25. A composition according to paragraph 24 wherein the hydrocolloid is selected from the group consisting of an alginate, a carrageenan, a pectin and a vegetable gum.

26. A composition according to paragraph 21 wherein the amount of glycerol oxidase is in the range of 10 to 10,000 units per kg flour.

27. A composition according to paragraph 21 or 26, comprising as the further dough additive a lipase in an amount which is in the range of 10 to 100,000 LUS per kg flour.

28. Use of a glycerol oxidase for improving the rheological properties of a flour dough and the quality of the finished product made from the dough.

29. Use according to paragraph 28 wherein the improvement of the rheological properties include that the resistance to extension of the dough in terms of the ratio between resistance to extension (height of curve, B) and the extensibility (length of curve, C), i.e. the B/C ratio, as measured by the AACC method 54-10 is increased by at least 10% relative to that of an otherwise similar dough not containing glycerol oxidase.

30. Use of a glycerol oxidase and a lipase in combination for improving the rheological properties of a flour dough and the quality of the finished product made from the dough.

31. Use according to paragraph 30 wherein the improvement of the rheological properties of the dough include that the resistance to extension of the dough in terms of the ratio between resistance to extension (height of curve, B) and the

extensibility (length of curve, C), i.e. the B/C ratio, as measured by the AACC method 54-10 is increased by at least 10% relative to that of an otherwise similar dough that does not contain glycerol oxidase.

32. Use according to paragraph 30 wherein the improvement of the quality of the finished product made from the dough is that the average pore diameter of the crumb of the bread made from the dough is reduced by at least 10%, relative to a bread which is made from a bread dough without addition of the lipase.

33. Use according to paragraph 30 wherein the improvement of the quality of the finished product made from the dough is that the pore homogeneity of the crumb of the bread made from the dough is increased by at least 5%, relative to a bread which is made from a bread dough without addition of the lipase.

34. Use according to paragraph 30 or 31 wherein the improvement of the rheological characteristics of the dough includes that the gluten index in the dough is increased by at least 5%, relative to a dough without addition of a lipase, the gluten index is determined by means of a Glutomatic 2200 apparatus.

INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

(PCT Rule 13*bis*)

A. The indications made below relate to the microorganism referred to in the description on page 37, line 6

B. IDENTIFICATION OF DEPOSIT — Further deposits are identified on an additional sheet ☐

Name of depositary institution: The National Collections of Industrial and Marine Bacteria Limited (NCIMB)

Address of depositary institution *(including postal code and country)*: 23 St. Machar Drive, Aberdeen, Scotland United Kingdom, AB2 1RY

Date of deposit	Accession Number
24 February 1997	NCIMB 40863

C. ADDITIONAL INDICATIONS *(leave blank if not applicable)* — This information is continued on an additional sheet ☐

As regards the respective Patent Offices of the respective designated states, the applicants request that a sample of the deposited microorganisms only be made available to an expert nominated by the requester until the date on which the patent is granted or the date on which the application has been refused or withdrawn or is deemed to be withdrawn.

D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE *(if the indications are not for all designated States)*

E. SEPARATE FURNISHING OF INDICATIONS *(leave blank if not applicable)*

The indications listed below will be submitted to the International Bureau later *(specify the general nature of the indications e.g., "Accession Number of Deposit")*

For receiving Office use only	For International Bureau use only
☒ This sheet was received with the international application	☐ This sheet was received by the International Bureau on:
Authorized officer: Jette Cordes Paulsen, Head Clerk	Authorized officer

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:
(A) NAME: DANISCO A/S
(B) STREET: Langebrogade 1
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(F) POSTAL CODE (ZIP): 1001 K
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(ii) TITLE OF INVENTION: Cloning and use of Lipase 3 gene from Aspergillus tubigensis

(iii) NUMBER OF SEQUENCES: 9

(iv) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30 (EPO)

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(v) FRAGMENT TYPE: N-terminal

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Aspergillus tubigensis

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

Ser Val Ser Thr Ser Thr Leu Asp Glu Leu Gln Leu Phe Ala Gln Trp
1 5 10 15

Ser Ala Ala Ala Tyr Xaa Ser Asn Asn
20 25

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 7 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Aspergillus tubigensis

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

Val His Thr Gly Phe Trp Lys
1 5

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 14 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Aspergillus tubigensis

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

Ala Trp Glu Ser Ala Ala Asp Glu Leu Thr Ser Lys Ile Lys
1 5 10

(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "Oligonucleotide"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

TTCCARAANC CNGTRTGNAC 20

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(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "Oligonucleotide"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

CARYTNTTYG CNCARTGG 18

(2) INFORMATION FOR SEQ ID NO: 6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 17 base pairs

(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "Oligonucleotide"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

GCVGCHSWYT CCCAVGC 17

(2) INFORMATION FOR SEQ ID NO: 7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 317 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "PCR fragment"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

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TCCAACTTGA CATGCACGGC CAACGCCTGT CCATCAGTCG AGGAGGCCAG TACCACGATG 120

CTGCTGGAGT TCGACCTGTA TGTCACTCAG ATCGCAGACA TAGAGCACAG CTAATTGAAC 180

AGGACGAACG ACTTTTGGAG GCACAGCCGG TTTCCTGGCC GCGGACAACA CCAACAAGCG 240

GCTCGTGGTC GCCTTCCGGG GAAGCAGCAC GATTGAGAAC TGGATTGCTA ATCYTGACTT 300

CATCCTGGRA GATAACG 317

(2) INFORMATION FOR SEQ ID NO: 8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1045 base pairs

(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Aspergillus tubigensis

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION:join(1..82, 135..300, 347..683, 737..1045)

(ix) FEATURE:
(A) NAME/KEY: sig_peptide
(B) LOCATION:1..81

(ix) FEATURE:
(A) NAME/KEY: mat_peptide
(B) LOCATION:join(82, 135..300, 347..683, 737..1042)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

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GGT GCT GCC GCG CCG GCA CCG CTT GCT GTG CGG A GTAGGTGTGC          92
Gly Ala Ala Ala Pro Ala Pro Leu Ala Val Arg
    -10                 -5

CCGATGTGAG ATGGTTGGAT AGCACTGATG AAGGGTGAAT AG  GT GTC TCG ACT   145
                                                Ser Val Ser Thr
                                                  1

TCC ACG TTG GAT GAG TTG CAA TTG TTC GCG CAA TGG TCT GCC GCA GCT   193
Ser Thr Leu Asp Glu Leu Gln Leu Phe Ala Gln Trp Ser Ala Ala Ala
  5                 10                  15                  20

TAT TGC TCG AAT AAT ATC GAC TCG AAA GAC TCC AAC TTG ACA TGC ACG   241
Tyr Cys Ser Asn Asn Ile Asp Ser Lys Asp Ser Asn Leu Thr Cys Thr
                25                  30                  35

GCC AAC GCC TGT CCA TCA GTC GAG GAG GCC AGT ACC ACG ATG CTG CTG   289
Ala Asn Ala Cys Pro Ser Val Glu Glu Ala Ser Thr Thr Met Leu Leu
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                40                  45                  50

    GAG TTC GAC CT  GTATGTCACT CAGATCGCAG ACATAGAGCA CAGCTAATTT        340
    Glu Phe Asp Leu
5            55

    GAACAG G ACG AAC GAC TTT GGA GGC ACA GCC GGT TTC CTG GCC GCG GAC   389
             Thr Asn Asp Phe Gly Gly Thr Ala Gly Phe Leu Ala Ala Asp
                         60                  65                  70
10
    AAC ACC AAC AAG CGG CTC GTG GTC GCC TTC CGG GGA AGC AGC ACG ATT   437
    Asn Thr Asn Lys Arg Leu Val Val Ala Phe Arg Gly Ser Ser Thr Ile
                    75                  80                  85

15  GAG AAC TGG ATT GCT AAT CTT GAC TTC ATC CTG GAA GAT AAC GAC GAC   485
    Glu Asn Trp Ile Ala Asn Leu Asp Phe Ile Leu Glu Asp Asn Asp Asp
                90                  95                  100

    CTC TGC ACC GGC TGC AAG GTC CAT ACT GGT TTC TGG AAG GCA TGG GAG   533
20  Leu Cys Thr Gly Cys Lys Val His Thr Gly Phe Trp Lys Ala Trp Glu
            105                 110                 115

    TCC GCT GCC GAC GAA CTG ACG AGC AAG ATC AAG TCT GCG ATG AGC ACG   581
    Ser Ala Ala Asp Glu Leu Thr Ser Lys Ile Lys Ser Ala Met Ser Thr
25      120                 125                 130

    TAT TCG GGC TAT ACC CTA TAC TTC ACC GGG CAC AGT TTG GGC GGC GCA   629
    Tyr Ser Gly Tyr Thr Leu Tyr Phe Thr Gly His Ser Leu Gly Gly Ala
    135                 140                 145                 150
30
    TTG GCT ACG CTG GGA GCG ACA GTT CTG CGA AAT GAC GGA TAT AGC GTT   677
    Leu Ala Thr Leu Gly Ala Thr Val Leu Arg Asn Asp Gly Tyr Ser Val
                    155                 160                 165

35  GAG CTG GTGAGTCCTT CACAAAGGTG ATGGAGCGAC AATCGGGAAC AGACAGTCAA    733
    Glu Leu

    TAG TAC ACC TAT GGA TGT CCT CGA ATC GGA AAC TAT GCG CTG GCT GAG   781
40      Tyr Thr Tyr Gly Cys Pro Arg Ile Gly Asn Tyr Ala Leu Ala Glu
            170                 175                 180

    CAT ATC ACC AGT CAG GGA TCT GGG GCC AAC TTC CGT GTT ACA CAC TTG   829
    His Ile Thr Ser Gln Gly Ser Gly Ala Asn Phe Arg Val Thr His Leu
45      185                 190                 195
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AAC GAC ATC GTC CCC CGG GTG CCA CCC ATG GAC TTT GGA TTC AGT CAG   877
Asn Asp Ile Val Pro Arg Val Pro Pro Met Asp Phe Gly Phe Ser Gln
200                 205                 210                 215

CCA AGT CCG GAA TAC TGG ATC ACC AGT GGC AAT GGA GCC AGT GTC ACG   925
Pro Ser Pro Glu Tyr Trp Ile Thr Ser Gly Asn Gly Ala Ser Val Thr
                220                 225                 230

GCG TCG GAT ATC GAA GTC ATC GAG GGA ATC AAT TCA ACG GCG GGA AAT   973
Ala Ser Asp Ile Glu Val Ile Glu Gly Ile Asn Ser Thr Ala Gly Asn
            235                 240                 245

GCA GGC GAA GCA ACG GTG AGC GTT GTG GCT CAC TTG TGG TAC TTT TTT  1021
Ala Gly Glu Ala Thr Val Ser Val Val Ala His Leu Trp Tyr Phe Phe
        250                 255                 260

GCG ATT TCC GAG TGC CTG CTA TAA                                  1045
Ala Ile Ser Glu Cys Leu Leu  *
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(2) INFORMATION FOR SEQ ID NO: 9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 297 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

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Gly Ala Ala Ala Pro Ala Pro Leu Ala Val Arg Ser Val Ser Thr Ser
    -10                 -5                  1                   5

Thr Leu Asp Glu Leu Gln Leu Phe Ala Gln Trp Ser Ala Ala Ala Tyr
                10                  15                  20

Cys Ser Asn Asn Ile Asp Ser Lys Asp Ser Asn Leu Thr Cys Thr Ala
            25                  30                  35

Asn Ala Cys Pro Ser Val Glu Glu Ala Ser Thr Thr Met Leu Leu Glu
```

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          40                  45                  50

Phe Asp Leu Thr Asn Asp Phe Gly Gly Thr Ala Gly Phe Leu Ala Ala
     55                  60                  65

Asp Asn Thr Asn Lys Arg Leu Val Val Ala Phe Arg Gly Ser Ser Thr
 70                  75                  80                  85

Ile Glu Asn Trp Ile Ala Asn Leu Asp Phe Ile Leu Glu Asp Asn Asp
                 90                  95                 100

Asp Leu Cys Thr Gly Cys Lys Val His Thr Gly Phe Trp Lys Ala Trp
            105                 110                 115

Glu Ser Ala Ala Asp Glu Leu Thr Ser Lys Ile Lys Ser Ala Met Ser
        120                 125                 130

Thr Tyr Ser Gly Tyr Thr Leu Tyr Phe Thr Gly His Ser Leu Gly Gly
    135                 140                 145

Ala Leu Ala Thr Leu Gly Ala Thr Val Leu Arg Asn Asp Gly Tyr Ser
150                 155                 160                 165

Val Glu Leu Tyr Thr Tyr Gly Cys Pro Arg Ile Gly Asn Tyr Ala Leu
                170                 175                 180

Ala Glu His Ile Thr Ser Gln Gly Ser Gly Ala Asn Phe Arg Val Thr
            185                 190                 195

His Leu Asn Asp Ile Val Pro Arg Val Pro Pro Met Asp Phe Gly Phe
        200                 205                 210

Ser Gln Pro Ser Pro Glu Tyr Trp Ile Thr Ser Gly Asn Gly Ala Ser
    215                 220                 225

Val Thr Ala Ser Asp Ile Glu Val Ile Glu Gly Ile Asn Ser Thr Ala
230                 235                 240                 245

Gly Asn Ala Gly Glu Ala Thr Val Ser Val Val Ala His Leu Trp Tyr
                250                 255                 260

Phe Phe Ala Ile Ser Glu Cys Leu Leu  *
            265                 270
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